$CD4^+$ T cell mediated chronic intestinal disease: immune regulation versus inflammation

Von der Gemeinsamen Naturwissenschaftlichen Fakultät
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr.rer.nat.)

genehmigte
Dissertation

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eingereicht am: 27. Oktober 2003
Disputation am: 28. Januar 2004

Danksagung

Die vorliegende Arbeit wurde an der Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, in der Abteilung Zellbiologie und Immunologie, Arbeitsgruppe Mukosale Immunologie, angefertigt.

Ich danke dem Mentor dieser Arbeit, Herrn Prof. Dr. J. Wehland, für die Betreuung des Promotionsverfahrens und die Übernahme des Hauptreferats. Ebenso danke ich Herrn Prof. Dr. S. Dübel für die Übernahme des Korreferats und Herrn Prof. Dr. N. Käufer für die Bereitschaft als Prüfer zur Verfügung zu stehen.

Mein besonderer Dank gilt Herrn Prof. Dr. J. Buer, in dessen Arbeitsgruppe diese Arbeit entstand, für die interessante Themenstellung sowie die großzügige Bereitstellung sämtlicher für diese Promotion benötigten Mittel. Sein offener, kollegialer Führungsstil gab mir die Freiräume, diese Arbeit nach meinen Vorstellungen erfolgreich zu gestalten.

Herzlichen Dank an meine Betreuerin Dr. Dunja Bruder und an Dr. Wiebke Hansen, die mit mir das Büro teilten. Dunja, Du hast mich von Anfang bis zum Ende dieser Arbeit immer und in jeder Hinsicht unterstützt. Mit Dir hat das Wort „Teamwork“ eine ganz besondere Bedeutung bekommen. Ihr habt durch Eure angenehme und doch so unterschiedliche Art nicht nur zu einem hervorragenden Arbeitsklima beigetragen, sondern auch jedes große Problem zu einem kleinen werden lassen. Danke!

Für die freundschaftliche Zusammenarbeit und angenehme Arbeitsatmosphäre bedanke ich mich weiterhin bei allen derzeitigen und ehemaligen Mitarbeitern der Arbeitsgruppe Mukosale Immunität.

Besonders bedanken möchte mich bei meinen Eltern, meinen Schwestern und Ulf, die mir alle auf ihre eigene, ganz besondere Weise zur Seite gestanden haben. Aber alle hatten einen Satz gemeinsam: „Du schaffst das schon“!

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Gemeinsamen Naturwissenschaftlichen Fakultät, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Templin, M.*; Westendorf, A.M.*; Gruber, A.D.; Prost-Kepper, M.; Hansen, W.; Lauber, J.; Liblau, R.S.; Gunzer, F.; Bruder, D. and Buer, J. Induction of chronic mucosal inflammation in mice by enterocyte-specific $CD4^+$ T cells. (Zur Veröffentlichung eingereicht); *****gleichberechtigte Erstautoren.

Tagungsbeiträge

Westendorf, A.M.; Lauber, J.; Gatzlaff, P.; Konieczny, M.P.J.; Schmidt, M.A.; Buer, J. and Bruder, D. Bacterial cell surface display of recombinant polypeptides including functional T cell epitopes by the AIDA autotransporter system (**Poster**). Joint Annual Meeting 2000 of the German and Dutch Society of Immunology, Düsseldorf, 2000.

Westendorf, A.M.; Gatzlaff, P.; Schmidt, M.A.; Buer, J. and Bruder, D. Modulation of mucosal immune response using bacterial cell surface display of a functional T cell epitope (**Vortrag**). Physiology of the mucosal immune response – Advanced course by Scuola Superiore d'Immmunologia Ruggero Ceppellini, Neapel, 2001.

Westendorf, A.M.; Geffers, R.; Templin, M.; Buer, J. and Bruder, D. $CD4^+$ T cell mediated chronic intestinal disease: immune regulation versus inflammation (**Poster**). 34^{th} Annual Meeting of the German Society of Immunology, Berlin, 2003.

Westendorf, A.M.; Geffers, R.; Templin M.; Buer, J. and Bruder, D. $CD4^+$ T cell mediated chronic intestinal disease: immune regulation versus inflammation. (**Vortrag**). 3rd Meeting of the European Mucosal Immunology Group, Berlin, 2003.

Zusätzliche Publikationen

Bruder, D.; Probst-Kepper, M.; Westendorf, A.M.; Geffers, R.; von Boehmer, H.; Buer, J. and Hansen, W. Neuropilin-1: A surface marker of regulatory T cells. (Zur Veröffentlichung eingereicht).

Bruder, D.; Westendorf, A.M.; Geffers, R.; Gruber, A.D.; Kreipe, H.H.; Enelow, R.I. and Buer, J. Pathogenesis of $CD4^+$ T cell mediated mucosal inflammation in an autoimmune mouse model of pulmonary disease. (Zur Veröffentlichung eingereicht).

Zusätzliche Tagungsbeiträge

Bruder, D.; Westendorf, A.M.; Gruber, A. and Buer, J. Mucosal antigen expression leads to lung-specific autoimmunity in a transgenic mouse model. (**Vortrag**). 33th Annual Meeting of the German Society of Immunology, Marburg, 2002.

Hansen, W.; Bruder, D.; Probst-Kepper, M.; Robert, G., Westendorf, A.M.; von Boehmer, H. and Buer, J. Neuropilin-1: A surface marker of regulatory T cells. (**Vortrag**). 34th Annual Meeting of the German Society of Immunology, Berlin, 2003.

Bruder, D.; Westendorf, A.M.; Geffers, R.; Gruber, A. and Buer, J. Pathogenesis of $CD4^+$ T cell mediated mucosal inflammation in an autoimmune mouse model of pulmonary disease. (**Poster**). 34th Annual Meeting of the German Society of Immunology, Berlin, 2003.

Bruder, D.; Westendorf, A.M.; Geffers, R.; Gruber, A. and Buer, J. Pathogenesis of $CD4^+$ T cell mediated mucosal inflammation in an autoimmune mouse model of pulmonary disease. (**Poster**). 3rd Meeting of the European Mucosal Immunology Group, Berlin, 2003.

Bruder, D.; Westendorf, A.M.; Geffers, R.; Gereke, M.; Gruber, A. and Buer, J. Toleranz versus Autoimmunität: Autoreaktive $CD4^+$ T-Zellen in der Pathogenese chronisch entzündlicher Erkrankungen der Lunge. (**Vortrag**). Tagung der Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie, Magdeburg, 2003.

CHAPTER I

Introduction

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Intestinal Immunity

Intestinal immunity is a relatively new term in relation to events and processes that regard human biology. Microorganisms inhabited the earth long before humans. The evolution of intestinal immunity was therefore influenced by its interaction with these microorganisms. The intestinal tract successfully handles the situation in a way better than any other defense system. It encounters an enormous load of antigens while maintaining a normal homeostatic environment. Most of these antigens are beneficial to the host, such as dietary antigens and symbiotic bacteria, and some are harmless, such as commensals. On the other hand, pathogenic microorganisms need to be recognized and eliminated before damage occurs. Whereas the systemic immune system elicits an aggressive immune response to exposure of any nonself antigens, the intestinal immune system needs to be more flexible. Antigens need to be sampled, processed, and presented in such a way that enables the elimination of pathogens and tolerance to nonpathogens. Therefore, the rules governing intestinal immunity differ from those observed in systemic immunity. The challenge of facing billions of bacteria, limitless dietary antigens, and the largest pool of lymphocytes in the body necessitated the development of unique cells, mediator, and regulatory processes. Cells of the gut-associated lymphoid tissue (GALT) include conventional cells of the innate and adaptive immune system such as B and T lymphocytes, macrophages and dendritic cells (DC), as well as non classical antigen-presenting cells (APC), such as intestinal epithelial cells (IEC), and finally the lymphocytes specific for the GALT, lamina propria lymphocytes (LPL) and intestinal epithelial lymphocytes (IEL). As consequence of their antigen-exposed environment, these cells have unique activation requirements, they secrete and are influenced by a special array of cytokines and mediators.

1 Gastrointestinal immune system

1.1 Barrier and unspecific defense mechanisms

The intestinal mucosa is the interface between the immune system and the massive antigenic load represented by the commensal and potentially pathogenic enteric bacteria (Hooper et al., 1998). A variety of mechanisms contribute to the ability of the gut to either react or remain tolerant to antigen present in the intestinal lumen. The epithelial cell layer forms a barrier against exposure to mucosal microflora and other mucosal antigens and thus plays a key role in regulation of mucosal immune responses. Crucial for an efficient barrier function are specialized adaptations of the intestine, including mucus secretion, tight junctions between epithelial cells, defensins and immunoglobulin (Ig) A. Mucus is produced as a thick layer along the intestinal membrane. The functions of these large-molecular-weight glycoproteins are multifold: trapping bacteria and viruses, preventing them from gaining initial access to the host and serving as a microenvironment for the accumulation of bacteriocidal and bacteriostatic chemical enzymes. Intestinal epithelial cells (IEC) form intercellular tight junctions that effectively restrict transepithelial movement of particulate and even hydrophilic molecules of molecular mass higher than 2.000 Da, thus preventing uncontrolled uptake of bacteria and many of their metabolites (Madara et al., 1992). However, the defense function of the intestinal epithelium is not limited to providing a barrier. Rather, the intestinal epithelium actively interacts with both microbes and immune cells via the secretion of cellular immune mediators. A specialized form of peptides secreted by IEC are defensins and trefoil peptides which exhibit direct antimicrobial activity (Ayabe et al., 2002; Wong et al., 1999). In addition, the intestinal epithelium transports products of bacteria and immune cells. For example, it has long been known that immunoglobulin A (IgA) produced by B cells is transcytosed by IEC into the intestinal lumen and that this IgA is protective for the host via neutralization of important molecules on the bacterial surface or binding and elimination of bacteria that already invaded the epithelium (Robinson et al., 2001).

1.2 The inductive sites for mucosal immune responses

1.2.1 Peyers's patches and M cells

Peyer's patches are macroscopic lymphoid aggregates that are located in the submucosa along the length of the small intestine. Mature Peyer's patches consist of collections of large B cell follicles and intervening T cell areas. The lymphoid areas are separated from the intestinal lumen by a single layer of columnar epithelial cells, known as the follicle-associated epithelium (FAE), and a more diffuse area immediately below the epithelium, known as the subepithelial dome (SED).

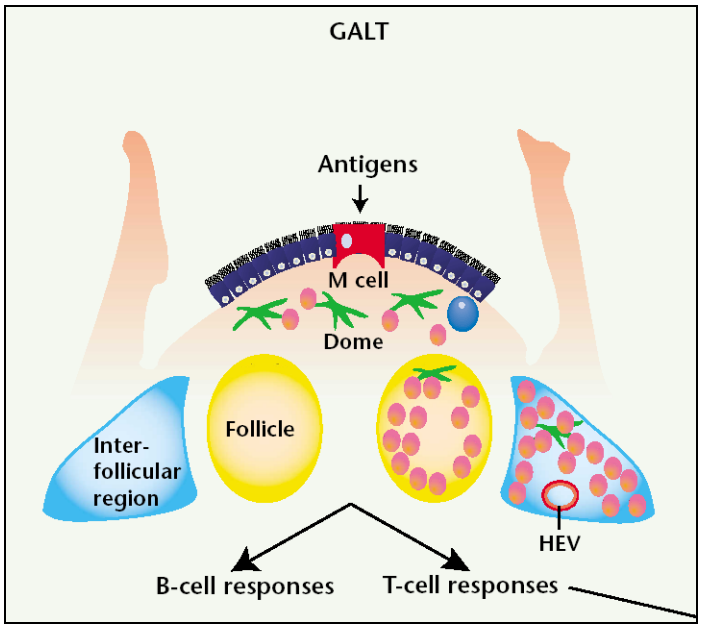


Figure 1: Inductive sites of the GALT. The GALT is the major inductive site of the gastrointestinal tract. The Payer's patches of the GALT consist of a follicle-associated epithelium with specialized M cells, a subepithelial dome overlying follicles, and interfollicular regions enriched in T cells (adapted from Neurath et al., 2002).

The FAE differs from the epithelium that covers the villus mucosa, as it has lower levels of digestive enzymes and a less pronounced brush border. The brush border is the surface layer of the normal small intestine that is comprised of small microvilli coated in a rich glycocalyx of mucus and other glycoproteins. Microvilli contain many of the digestive enzymes and transporter systems that are involved in the metabolism and uptake of dietary material. The brush border provides a large surface area for absorption. The FAE is infiltrated by large numbers of B cells, T cells, macrophages and dendritic cells and the most notable feature of the FAE is the presence of microfold (M) cells, which are specialized enterocytes that lack surface microvilli and the normal thick layer

of mucus. Uptake and presentation of antigens to naïve T and B cells to induce an adequate immune response is the primary function of the M cells in the mucosa.
Following ingestion, antigens and microorganisms are transported from the gut lumen to the dome region through these specialized M cells. Here they encounter APCs such as DCs leading to cognate interactions between APCs and T cells. DCs can also migrate to the interfollicular regions enriched with T cells and containing high endothelial venules (HEV) and efferent lymphatics to initiate an immune response upon antigen uptake.

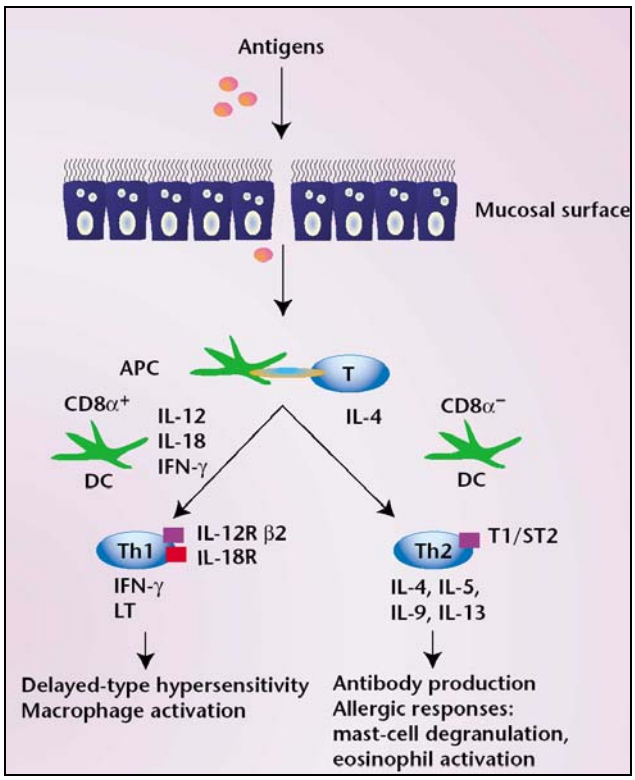


Figure 2: Immune response in the intestine. Antigens can be presented to T cells by DCs. In the normal gut immune system, immature DCs seem to preferentially induce T_{reg} and T_H3 T cell responses. In the presence of cytokines such as IL-12 and IFN-α produced by $CD8\alpha^+$ DCs, T cells can differentiate into T_H1 effector cells, whereas IL-4 can induce T_H2 T cell differentiation. T_H1 cells express the IL-12 receptor β2 chain and the IL-18 receptor, T_H2 cells express an IL-1-like molecule that appears to regulate T_H2 effector functions both in the peripheral and the mucosal immune system (adapted from Neurath et al., 2002).

Following induction in the GALT, mature lymphocytes leave the inductive sides and migrate to the effector sides such as the lamina propria where they can induce pro-inflammatory as well as suppressive immune responses. Among the pro-inflammatory signals cytokines produced by mucosal T_H1 and T_H2 effector cells have a central regulatory role.

1.3 The effector sites for mucosal immune responses

1.3.1 Intestinal epithelial cells (IEC)

Besides of professional antigen presenting cells like DCs, macrophages and B cells, non-professional antigen presenting cells exist in the intestine. Epithelial cells of the GALT play an important role as accessory cells in both B and T cell mucosal immune functions. Whereas their role in B cell mediated immunity, which is the transport of IgA synthesized by plasma cells in the lamina propria to the mucosal surface, is well characterized (Mestecky & Russell, 1991), the role of epithelial cells in mucosal T cell function is not yet fully understood. Since the 1980's it became clear that IEC express several surface molecules that are involved in antigen presentation, such as MHC molecules, CD1d or CD86. However, in contrast to professional APC, IEC are not equipped with the complete setting of antigen-presenting and costimulatory molecules. Therefore, they might be able of providing lymphocytes with the TCR stimulatory signal without costimulation, a situation normally resulting in anergy rather than T cell activation (Chen at al., 1995). It is well established that most of the LPL, as well as the IEL, are memory cells requiring less or even no costimulation to get activated. More important, it was discovered that in addition to classical MHC class I and II molecules, IEC express several unique costimulatory molecules either constitutively or during inflammation. Under normal conditions IEC do not express B7-1, B7-2 or CD40, but do express LFA-3 (Framson et al., 1999) and gp180 (Yio & Mayer 1997). In contrast, in inflammatory bowel disease IEC are induced to express B7-2 as well as infected IEC do express intercellular adhesion molecule-1 (ICAM-1) (Huang et al., 1996). Taken together, the localization of IEC separating antigens from lymphocytes, the expression of surface molecules required for lymphocyte activation and costimulation, and the ability to interact with LPL as well as IEL make the IEC a prime candidate for antigen presentation in the mucosal immune system.

For the interaction of IEC with T cells, Dotan & Mayer (Fig. 3) developed a model suggesting that luminal antigens derived from food or bacteria may be internalized via the apical epithelial surface (Dotan & Mayer, 2003). During inflammatory processes, paracellular transport of antigens and presentation by basolateral surface molecules occurs. The amount and type of antigen, as well as the combination of antigen-presenting molecules with costimulatory molecules determines the population of T cells that will expand. $CD8^+$ IEL and LPL may be stimulated by classical MHC class I

molecules. Stimulation by class I-like molecules, such as the complex gp180:CD1d and MICA/MICB, may also occur. Here, the antigen presented in the IEC:$CD8^+$ T cell interaction is of nonpeptide origin.

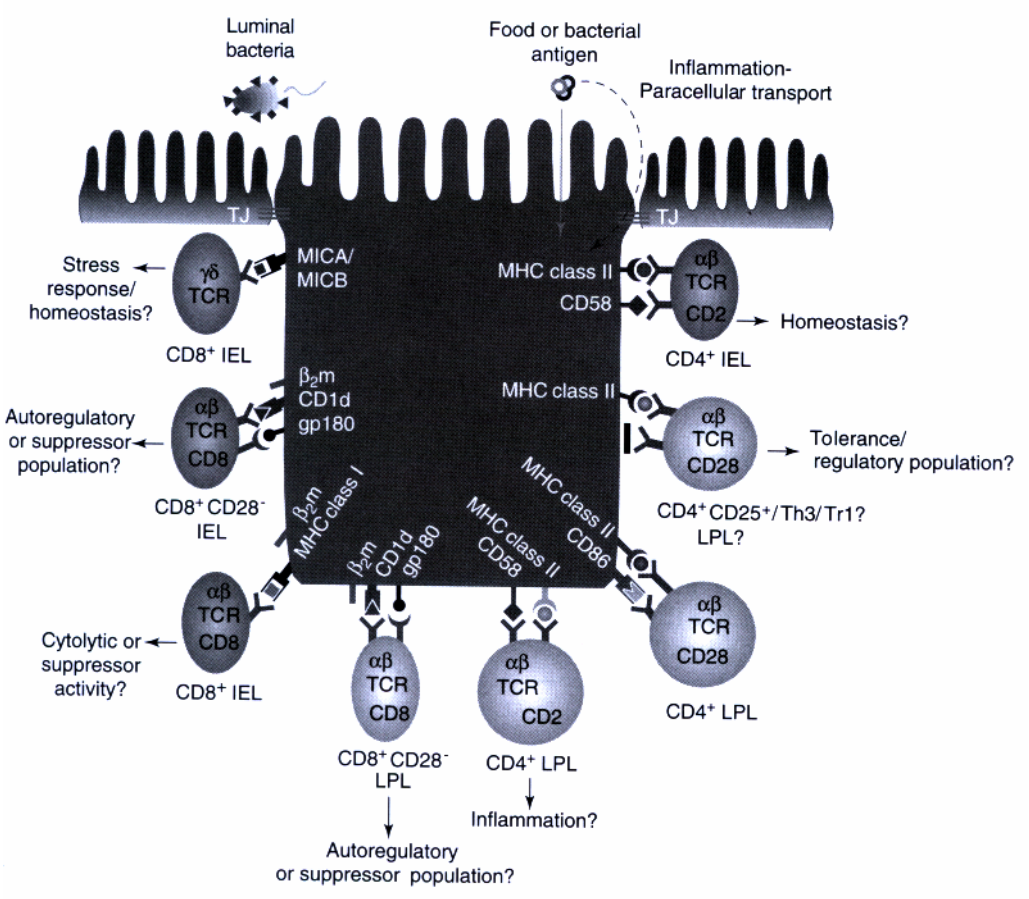


Figure 3: The interaction of IEC with T cells. IEC express a variety of surface molecules relevant for antigen presentation and stimulation of T cells. Luminal antigens derived from food or bacteria may be internalized via the apical surface. In the presence of inflammation, paracellular transport of antigens and presentation by basolateral surface molecules occur. The amount and type of antigen, as well as the combination of antigen-presenting molecules with costimulatory molecules, determine the population of T cells that will expand (adapted from Dotan & Mayer, 2003).

When presented by CD1d, data suggested bacterial-derived phospholipids as antigens. $CD8^+$ T cells activated by IEC have a suppressor activity and may function in regulating mucosal homeostasis. Peptide antigens can be presented to $CD4^+$ T cells by MHC class II molecules, which are constitutively expressed on IEC. Different $CD4^+$ T cell

populations may expand when the antigen is taken up via the apical or the basolateral surface. In normal mucosal homeostasis, regulatory $CD4^+$ T cells, activated via MHC class II without costimulation, may contribute to controlled inflammation. In inflammatory states, up-regulation of MHC class II as well as costimulatory molecules such as B7-2 on IEC may promote the expansion of T_H1/T_H2 cells and contribute to uncontrolled inflammation.

1.3.2 Chemokine/Cytokine secretion by IEC

The IEC show a variety of functions in mucosal immune homeostasis. Their functions as barrier between the antigenic load and the mucosal immune system and as a nonprofessional APC have described in paragraph 1.1 and 1.3.1. In addition, IEC secrete mediators that influence cells in their vicinity. Several chemokines and cytokines have recently been shown to be expressed by IEC. IEC-derived chemokines can induce the migration of inflammatory cells towards the epithelium. Therefore, their production by IEC demonstrate the potential for active participation in intestinal innate and adaptive immune responses.

The main chemokines produced by IEC are CXC chemokines, monokine induced by IFN-γ (MIG), IFN-γ inducible protein 10 (IP-10), and interferon-inducible T-cell α-chemoattractant (I-TAC) (Dwinel et al., 2001; Shibahara et al., 2001). The receptors of these chemokines are expressed on IEL indicating a strong correlation between IEC and migration of IEL into the epithelium. A CC chemokine that attracts immature DCs, macrophage inflammatory protein-3α (MIP-3α), is also produced by IEC (Neutra et al., 2001). Besides chemokines, IEC produce cytokines like IL-7 and IL-15, cytokines with growth factor activity for $\gamma\delta TCR^+$ and $CD8^+$ T cells in the mucosa (Reinecker & Podolsky, 1995). Additionally, cytokines typically expressed by IEC are TGF-α and -β, IL-6, TNF-α, and IL-10 (Podolsky, 1997; Taylor et al., 1998; Hogan et al., 2001). Furthermore, the IEC express the receptors for these cytokines, which therefore seem to be involved in autocrine activation pathways of IEC.

1.3.3 Lymphocytes in the GALT

As mentioned above, two distinct lymphocyte populations do exist in the GALT, namely the intraepithelial lymphocytes and lamina propria lymphocytes which are

separated by the thin basement membrane between the epithelium and the Lamina propia. Both are different from systemic lymphocytes with regard to phenotype and activation requirements, also there are differences between those two population with respect to phenotype, function and cytokine secretion.

1.3.3.1 Intraepithelial lymphocytes (IEL)

IEL are among the most intriguing lymphocytes that exist. It is surprising that despite their numbers (epithelial lymphocytes are one of the major lymphocyte populations in the body) and the fact that they are the focus of intense research, their function is as yet poorly defined. This is attributed in part to difficulties in their isolation and to the intrinsic heterogeneity of IEL subsets. Virtually all IEL are T cells, most of which are $CD8^+$ (~ 70 %), at least in the small bowel. In contrast to their systemic counterparts, the majority of $CD8^+$ IEL in young mice expresses the CD8αα homodimer. Two phenotypes that are rare in circulation are existing in the IEL population: $CD4^-CD8^-$ double negative (~ 10 %) and $CD4^+CD8\alpha\alpha^+$ (5-10 %) T cells. Another unique characteristic of IEL is their TCR usage, either αβ or γδ. Since different TCR phenotypes are associated with different functional characteristics, Hayday et al. (2001) suggested a classification of type a and type b IEL, to simplify characterization. According to this classification, type a IEL are $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ and their proportion in the human small and large intestine is between 50 % and 100 %, respectively. Type a IEL are cytolytic cells with an oligoclonal repertoire that partially overlaps that of the lamina propria and thoracic duct $CD8^+$ T cells. This observations supports the hypothesis that type a IEL are primed in the mucosal-associated lymphoid tissue (MALT), migrate via the MLN and the thoracic duct to systemic circulation, and home back to the LP, from where they pass into the epithelium. In this regard, they express the integrin $\alpha_4\beta_7$, which binds to the mucosal addressin MAdCAM-1. Homing is also directed by chemokines expressed by the IEC such as IP-10, MIG and I-TAC (Dwinel et al., 2001; Shibahara et al., 2001). IEL express CXCR3, the cognate receptor for these chemokines, as well as CCR9, the receptor for TECK that is expressed in small bowel IEC (Zabel et al., 1999). Furthermore, almost all IEL express $\alpha_E\beta_7$. This integrin binds to E-cadherin on IEC and may lead to the accumulation of IEL within the epithelium.

Type b IEL are $TCR\alpha\beta^+$ $CD8\alpha\alpha^+$, TCRγδ $CD8\alpha\alpha^+$, and TCRγδ "double negative" T cells. They show a different gene expression for T cell maturation markers, as failing to express CD2, CD28 and CD5 (reviewed in Hayday et al., 2001). They differ also by

MHC restriction, as type a IEL being conventionally restricted while type b IEL do not react in a classical way to their specific antigen in the MHC restriction element. Type b IEL are less MHC dependent and may develop in athymic mice, leading to the hypothesis that these cells may be auto-reactive, regulatory cells positively selected in the periphery. Current data suggested that type b IEL may have a role in promoting epithelial repair and healing as well as in eliminating infected or transformed epithelial cells.

1.3.3.2 Lamina propria lymphocytes (LPL)

Another lymphocyte population of the intestinal immune system resides in the lamina propria. LPL are a very heterogeneous group of T and B cells. In contrast to IEL, the LP CD4:CD8 T cell ratio is similar to that in the blood, and they express the αβTCR. Similar to IEL, most of the LPL are memory cells exhibiting an activated phenotype. Interestingly, when stimulated via the TCR their responses are poor, and they seem to depend on CD2/CD28-mediated stimulation to proliferate and secrete cytokines (Boirivant et al., 1999; Targan et al., 1995). The propensity for stimulation via the CD2 pathway may be one explanation for the increased tendency towards apoptotic cell death in comparison to peripheral blood lymphocytes. The increased susceptibility to apoptosis may be related to the fact that the vast majority of LPL express FAS antigen and a subset also FAS ligand (DeMaria et al., 1996). Not only more LPL than their peripheral counterparts are FAS positive, also upon FAS ligation, cell death is induced more effectively in LPL, suggesting that they are “death prone” (DeMaria et al., 1996).
The significance of LPL preprogrammed cell death for intestinal homeostasis is seen in conditions in which this homeostasis is disturbed. In inflammatory bowel disease, mucosal inflammation is associated with decreased sensitivity of LPL to cell death induced by FAS ligation or by other cell-death inducers such as deprivation of IL-2 and exposure to nitric oxide. Differences between normal LPL and those isolated from inflamed mucosa show that apoptosis-associated genes such as *bax* and *bcl-2* are differentially expressed in normal versus inflamed mucosa. Specifically, increased expression of the anti-apoptitic BCL-2 protein, increased Bcl/Bax ratio in the mucosa, and decreased Bax expression in LPL were reported in Crohn’s disease (Ina et al. 1999.; Itoh et al., 2001). This suggests that normal LPL represent an activated, apoptosis-prone population and that dysregulation of the death propensity may leads to intestinal inflammation. There are inconsistent reports about the cytokine secretion of LPL.

However, it has been shown that IFN-γ is produced by LPL both in the normal state and in inflammatory conditions. Other cytokines produced under various conditions and stimuli are IL-5 in Ulcerative colitis and TNF-α in Crohn's disease (Camoglio et al., 1998; Fuss et al., 1996; Murch et al., 1993; Reinecker et al., 1993; Samoilova et al., 1998). The targets for these cytokines are other immune cells like $CD4^+$ T cells and macrophages, the IEC themselves, and even endothelial cells. A cytokine network is thus created in which activated LPL activate additional cells in the LP, thereby regulating or dysregulating mucosal homeostasis.

1.3.4 Cytokine regulation of the mucosal immune response via mucosal T cells

The differentiation into different T helper cell (T_H) and T regulatory (T_{reg}) cell cytokine response is a reasonable framework for describing the immune reactivity of systemic lymphoid tissues. Antigens, such as those derived from the microbial flora of the gut, are continuously sampled by M cells of the Peyer's patches. The antigens are interpreted by APC which direct the differentiation of naïve $CD4^+$ T_H0 cells to one of several states of polarized cytokine production under the influence of cytokines and their associated signaling pathways (Fig. 4).

T_H1 cells secrete pro-inflammatory cytokines such as IFN-γ, IL-2, and TNF-α; T_H2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13, and promote IgA expression and other immunoglobulin isotypes. T_H3 cells secrete TGF-β and T_{reg} cells predominantly produce IL-10. Many of the functions of T cells in the gastrointestinal immune system are mediated by secreted cytokines. Recently, it has been shown that lamina propria T cells are producing higher levels of IL-10 compared to peripheral blood lymphocytes (Braunstein et al., 1997; Autschbach et al., 1998). It seems that IL-10 secreting regulatory T cells inhibit T_H1 activation and that IL-10 produced in the intestine acts on macrophages to prevent their activation and the induction of pro-inflammatory cytokines, thereby inhibiting the recruitment of T cells to the intestine. It has also been shown that regulatory T cells, induced by oral antigen uptake, have characteristics of T_H2 or T_H3 cells (Weiner, 1997; Strobel & Mowat, 1998). On the other hand, it has been demonstrated in a TCR transgenic mouse model that continuous feeding of low dose antigen induces a T_H1 cytokine response (Marth et al., 2000). In contrast, recent data revealed that T_H1 cytokine production may not only have a pro-inflammatory effect as it

also can be protective in the immune regulation of certain infectious or autoimmune diseases (Dalton et al., 2000).

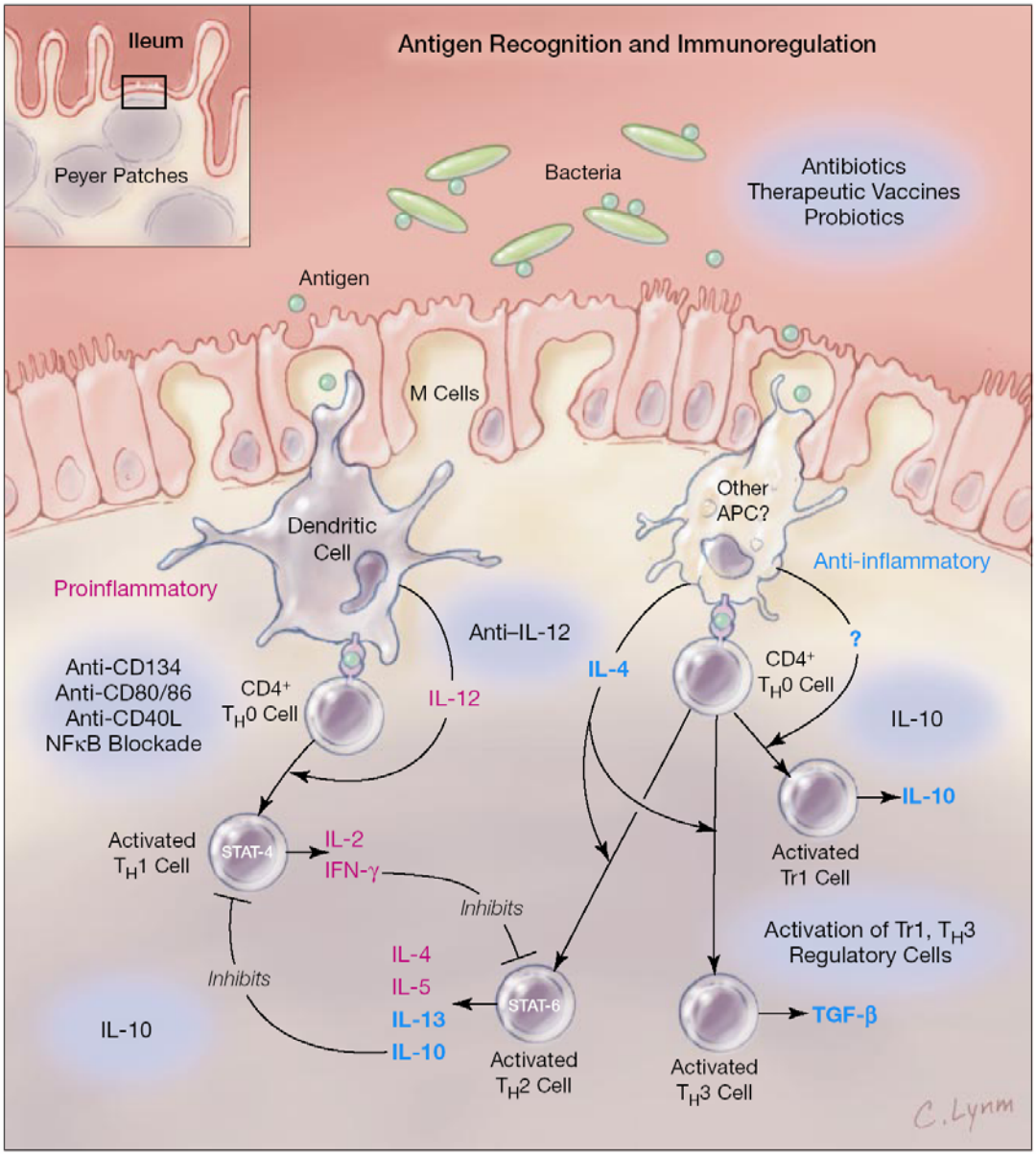


Figure 4: Antigen uptake and recognition by CD4$^+$ T cells in the intestine. Antigen might pass the epithelium through the M cells and after transfer to local DCs, might than be presented directly to naïve T_H0 cells, which directs the differentiation of the naïve T_H0 cells into one of several state of polarized cytokine production under the influence of cytokines and their associated intracellular pathways. Under normal conditions, a balance is established between generation of pro-inflammatory T_H1/T_H2 and anti-inflammatory $Tr1/T_H3$ cells. These activated cells spread widely via the lymphatic system to the lamina propria and the epithelium of the intestine. IL, interleukin; NFκB, Nuclear factor kappa B; IFN, interferon; TGF, tumor growth factor; Tr1; regulatory; STAT, signal transducer and activator of transcription (adapted from Blumberg & Strober, 2001).

In addition to TCR stimulation by MHC/peptide/TCR crosslinking, T cells require a second signal in form of costimulation for complete activation. In the lymphoid system, denditric cells express high levels of costimulatory molecules, and these are up-regulated upon maturation. In the healthy mucosa, lamina propria antigen-presenting cells provide only low levels of costimulatory signals (Qiao et al., 1996), but this second signal is up-regulated in inflammatory bowel disease (Rogler et al., 1999). Several reports suggest that the level and type of costimulation influences the naïve $CD4^+$ T_H0 cell to develop a T_H1 or T_H2 phenotype. The costimulatory molecule involved in this processes are e.g. CD40, CD40L, CD44v7 or B7-1/2 (Wittig et al., 2000, 1998, 1999; Kuchroo et al., 1995; Rulifson et al., 1997). However, it will be important to determine whether differences in the expression of costimulatory molecules in mucosal inductive sites regulate the intestinal immune response to mucosal antigens.

2 Intestinal micoflora

The gut is the natural habitat for a very diverse and dynamic bacterial community. The relevance and effect of resident bacteria on the host's physiology and pathology has been well documented. Many species of bacteria have evolved and adapted to live and grow in the intestine. The intestinal habitat of an individual contains 300 - 500 different species of bacteria (Simon&Gorbach, 1984; Borriello, 1986) and the number of microbial cells within the gut lumen is about 10 times higher than the number of eukaryotic cells in the human body (Bengmark, 1998). Several hundred grams of bacteria living within the gut lumen affect the host immune homeostasis. Some of these bacteria are pathogenic and can be a source of infection and sepsis under certain circumstances - for example if the integrity of the intestinal barrier is physically or functionally broken. However, the constant interaction between the host and its microbial guest can infer important health benefits to the host (Salminen et al., 1998).

2.1 Main functions of the microflora in health

Use of animals bred under germ-free conditions has provided important information about the effect of the microbial community of the gut on host physiology and pathology (Falk et al., 1998). Such studies suggest that the microflora has important and specific metabolic, trophic and protective functions.

A major metabolic function of the microflora is the fermentation of non-digestible dietary residue and endogenous mucus produced by epithelial cells (Roberfroid et al., 1995). Gene diversity in the microbial community provides various enzymes and biochemical pathways that are distinct from the host's own constitutive resources. Overall outcomes of this complex metabolic activity are recovery of metabolic energy and absorbable substrates for the host and in symbiosis supply of energy and nutritive products for bacterial growth and proliferation. The main topics of metabolic functions are in summary the salvage of energy as short-chain fatty acids, the production of vitamin K and the absorption of ions.

Possibly the most important role of short-chain fatty acids on gut physiology is their trophic effect on the intestinal epithelium. The rate of production of crypt cells is

reduced in the colon of rats bred in germ-free environments, and their crypts contain fewer cells than those colonized by conventional flora, suggesting that intraluminal bacteria effect cell proliferation in the gut (Alam et al., 1994). Additionally, the differentiation of epithelial cells is greatly affected by interactions with resident bacteria (Hooper et al., 2001). All major short-chain fatty acids stimulate epithelial cell proliferation and differentiation in the large and small bowel *in vivo* (Frankel et al., 1994). However, the microflora is not only important for the developing structure of the gut, as it has been shown that the interaction between gut and bacteria also influences the host immunity. The intestinal mucosa is the main interface between the immune system and the external environment. Thus, the gut-associated lymphoid tissue contains the largest pool of immunocompetent cells in the body (Brandzeag et al., 1989). The dialog between host and bacteria at the mucosal surface seems to play a part in development of a competent immune system. Animals bred in a germ-free environment have low densities of lymphoid cells in the gut mucosa, specialized follicle structures are small, and circulating concentration of immunoglobulin in the blood are low (Butler et al., 2000). Microbial colonization of the gastrointestinal tract affects the composition of gut-associated lymphoid tissue. Immediately after exposure to luminal microbes, the number of IEL expands greatly (Helgeland at al., 1996), germinal centers with immunoglobulin producing cells arise rapidly in follicles and the lamina propria (Cebra et al., 1998), and the concentration of immunoglobulin increases substantially in the serum (Butler et al., 2000). According to these results, the microflora is very necessary for the development of an intact, complete intestinal immune system.

Resident bacteria are the crucial line of resistance to colonization by exogenous microbes and, therefore are highly relevant in prevention of invasion of tissue by pathogens. Germ-free animals are extraordinary susceptible to infections (Taguchi et al., 2002). Several mechanisms have been implicated in the barrier effect. *In vitro*, bacteria compete for attachment sites in the brush border of intestinal epithelial cells (Bernet et al., 1994). Adherent non-pathogenic bacteria can prevent attachment and subsequent entry of pathogenic enteroinvasive bacteria into epithelial cells. Furthermore, bacteria can inhibit the growth of their competitors by producing antimicrobial substances (Brook, 1999; Lievin et al., 2000). However, resident bacteria are the first line of defense against exogenous pathogenic microbes.

2.2 Intestinal microflora in disease

Besides all positive characteristics of intestinal bacteria, gut flora might also be an essential factor in certain pathological disorders, including inflammatory bowel disease (IBD). Resident bacterial flora has been suggested to be an important factor in driving the inflammatory processes in human inflammatory bowel disease (Shanahan, 2001). In patients with IBD, intestinal T lymphocytes are hyperreactive against bacterial antigens, and Pizer et al. (1991) suggested that local tolerance mechanisms are abrogated in such patients. Moreover, patients with IBD have higher amounts of bacteria attached to their epithelial surface than healthy people (Swidsinski et al., 2002). Thus, uncontrolled activation of the intestinal immune system by elements of the flora could be a key event in the pathophysiology of IBD.

The idea that resident bacteria of the normal flora are involved in intestinal mucosal inflammation is supported by data from animal studies. Treatment with wide-spectrum antibiotics has been shown to mitigate mucosal inflammation in rats and mice with IBD (Videla et al., 1994). In general, experimental inflammation does not develop when mice are kept in germ-free environment (Sartor, 1997). The mucosal microflora is also required to initiate or maintain the inflammatory process, presumably by providing one or more antigens or costimulatory factors that drive the immune response in a genetically susceptible host. However, despite extensive research, so far no specific pathogenic microorganism has been shown to be directly associated with any of these models. In addition, it is known that antigens from most resident bacteria do not take part in the disease process, there is little evidence that pathogenic antigens come from a single organism, or even a restricted group of organisms (Cong et al., 1998). Furthermore, it is possible to induce disease in various models of mucosal inflammation by the introduction of a single microorganism into an otherwise germ-free host (Tab. 1). Although this shows that mucosal inflammation can be caused by a limited set of antigens, it does not imply that only the organism introduced causes disease in the model used or in any other model.

Table 1: Bacteria related models of inflammatory bowel disease

Animal model	SPF	Germfree	Bacteria
$CD4^{+}CD45RB^{high}$ SCID transfer	Colitis	No Colitis	*B. vulgaris*
IL-10 KO	Colitis, gastritis	No inflammation	Not *B. vulgaris*
IL-2 KO	Colitis,gastritis, hepatitis	Attenuated inflammation	ND
TCR KO	Colitis	No inflammation	ND

(adapted from Sator, 1995)

Finally not all members of the microflora necessarily represent pathogens in IBD. Evidence has recently emerged that a class of microorganisms, collectively described as 'probiotics', prevent rather than induce inflammation (Madson et al., 1999).

2.3 Probiotics

Probiotics are living microorganisms that have a beneficial effect on health by positively affecting the microbial environment. Probiotic candidates are usually *lactobacilli* or *bifidobacter*, but *E.coli* and other species have also been used to study probiotic effects. Probiotics are usually given as food supplements either alone or in combination with certain dietary polysaccharides that might independently affect the enteric flora. Probiotic treatment seems to be effective in patients with inflammatory bowel disease, and trials in animals with Crohn's disease have been encouraging (Shanahan, 2002; Shanahan 2001; Campieri et al., 1998). A change in the enteric flora might also contributes to the therapeutic effect of elemental and polymeric diets in patients with Crohn's disease.

Possible mechanisms of probiotic action in inflammatory bowel disease include the production of antimicrobial factors, competitive interaction with pathogens, and signaling with the epithelium (Shanahan, 2000). Epithelial signaling by some non pathogenic bacteria might have an anti-inflammatory effect by blocking degradation of IκB, which inhibits NF-κB (Neish et al., 2000). Probiotic effects *in vivo* have been shown to include changes in the permeability of the intestine and in function of the mucosal immune system. However, use of genetically engineered probiotic organisms should extend the scope of probiotic action to include localized delivery of anti-

inflammatory and other biologically relevant molecules to the inflamed mucosa (Steidler et al., 2000; Shanhan, 2000). As an example, food-grade *Lactococcus lactis* has been engineered to secrete IL-10 and was therapeutically effective when given intragastrically to mice with IBD (Steidler et al., 2000). According to this beneficial abilities, probiotics are predestined to be used as carrier organisms for gut focused drug specific therapy of IBD, but the use of the therapeutic potential of probiotics is likely to require more detailed understanding of the normal intestinal microflora.

3 Homeostasis of the intestinal immune system

The mucosal immune system has the crucial role in maintaining the balance between defense against pathogens and accommodation of non-pathogenic resident bacteria as well as the many potentially immunogenic dietary proteins (mucosal tolerance). In contrast to DCs in the systemic immune system, mucosal DCs seem to preferentially induce T_{reg} cells (Harrison & Hafler, 2000; Faria & Weiner, 1999; Viney et al., 1998; Weiner, 2001). This properties of the mucosal immune system might be due to the mucosal milieu, with its high concentration of anti-inflammatory cytokines, such as TGF-β, IL-4 and IL-10. In addition intestinal epithelial cells express MHC class II molecules (Mayrhofer & Schon-Hegrad, 1983) and have been shown to process and present antigen to primed $CD4^+$ T cells (Meyer & Shlien, 1987; Kaiserlian et al., 1989), but they might not be professional antigen-presenting cells in the sense that they do not provide appropriate costimulatory signals for the induction of fully competent immune-effector cells. Therefore, antigen presentation by these cells might also lead to the development of T_{reg} cell.

In the intestinal immune system different types of regulatory T cells with specific regulatory functions develop. First to mention the T_H3 cells, a population of $CD4^+$ T cells that produce transforming growth factor-β (TGF-β) and can be generated by repeated restimulation of mesenteric lymph node or splenic lymphocytes from mice that have been fed low dose of antigen for oral tolerance induction. Similar cells have been identified directly *in vivo* (Miller et al., 1992). A second population of regulatory T cells are the Tr1 cells. This population of $CD4^+$ T cells produce high amounts of IL-10. They can be generated *in vitro* in the presence of antigen, IL-10, IL-15 and/or type I interferon. These cells have not been isolated following oral tolerance induction *in vivo*, but have been shown to confer bystander suppression in experimental colitis in mice that have been fed with antigen (Groux et al., 1997). The third T_{reg} population is represented by intrathymic derived regulatory $CD4^+CD25^+$ T cells showing a potent ability to prevent auto-reactivity *in vivo*. Although few reports have described the induction of these cells by specific antigen in the periphery, one study has identified ovalbumin-specific $CD4^+CD25^+$ T cells with regulatory activity after feeding tolerogenic doses of antigen to mice (Thorstenson & Khoruts, 2001) and Walker et al. (2003) published a model in which T_{reg} population dynamics are shaped by the local antigenic environment.

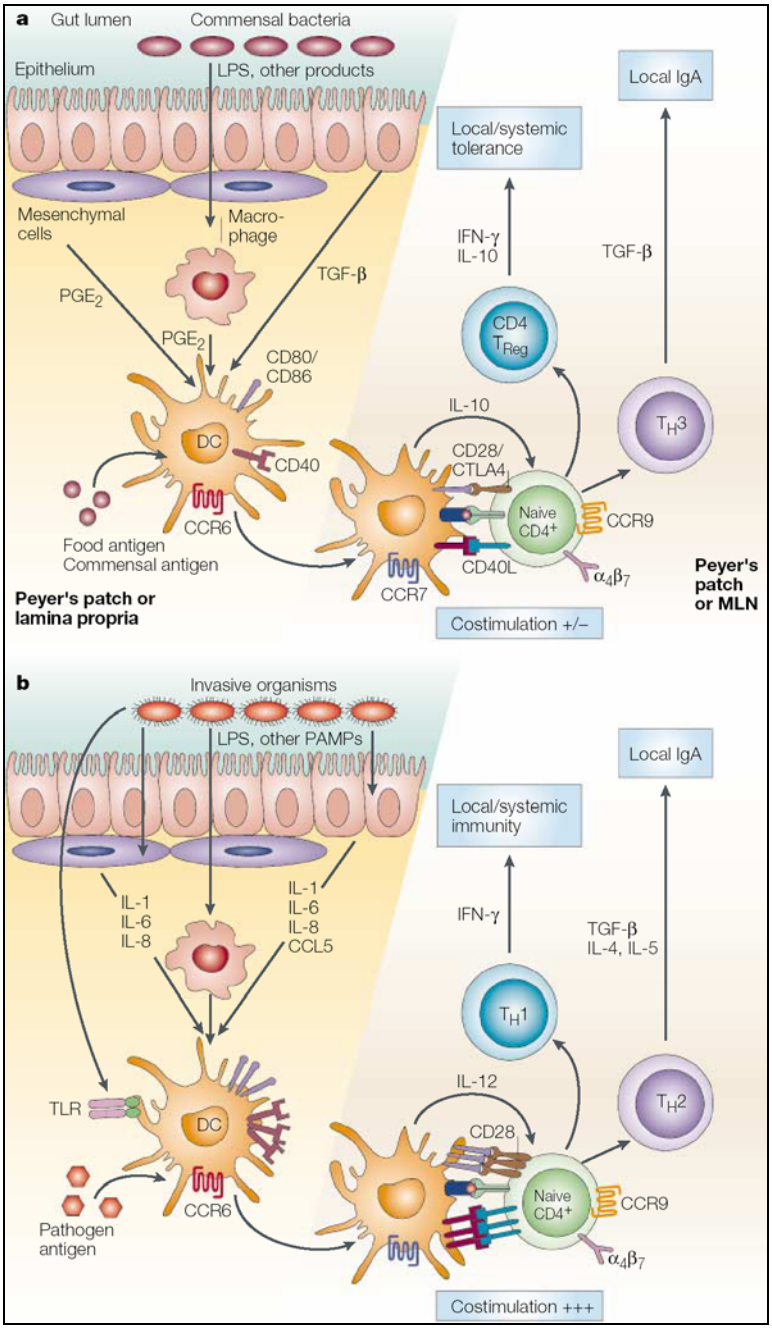


Figure 5: Proposed role of the intestinal microenvironment in polarizing immune functions. (a) Food proteins and products of commensal bacteria are taken up by DCs. In the absence of inflammation prostaglandin PGE_2, TGF-β, and IL-10 production result in a partial maturation of DCs in the Peyer's patch or lamina popria. The antigen is than presented to naïve $CD4^+$ T cells in the MLN or Peyer's patch. These cells can differentiate into regulatory T cells, which produce IL-10 and/or T_H3 cells, which produce TGF-β with die induction of local IgA production, systemic tolerance and local immune homeostasis. **(b)** By the uptake of pathogens, local inflammation is induced by effects of pathogen products mediated through Toll-like receptors that are expressed by mesenchymal cells, macro-phages and epithelial cells. As result, DCs in the Peyer's patch or lamina propria mature completely after antigen uptake and produce IL-12. After migrating to the MLN, these DCs prime gut-homing T_H1 cells, which produce INF-γ and cause further inflammation. CCR, CC-chemokine receptor; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular pattern (adapted from Mowat, 2003).

$CD8^+$ suppressor T cells represent the first population of regulatory T cells identified. They have been thought to be involved in oral tolerance induction (Mowat, 1987), but their functions and characteristics have not been clearly defined. T cells expressing a γδ TCR represent an additional population of regulatory T cells. Several studies in knock-out mice indicate that these cells play an important role in some models of oral tolerance. Indeed, tolerance can be transferred to untreated mice by injection of γδ T cells isolated from orally tolerized mice (Ke et al., 1997).

A model for the induction of regulatory T cells in the intestinal environment is shown in Fig. 5a. Food proteins and products of commensal bacteria are taken up by DCs in the absence of inflammation; prostaglandin E2 (PGE2), TGF-β and perhaps IL-10 results in the partial maturation of DCs in the Peyer's patches or lamina propria. The antigen is then presented to naïve $CD4^+$ T cells in the MLN or Peyer's patches. These T cells differentiate into regulatory T cells, which produce IL-10 and IFN-γ, and/or T_H3 cells, which produce TGF-β. The immunological consequences are local IgA production, systemic tolerance and local immune homeostasis. In Fig. 5b the situation after pathogen contact in the intestine is shown. The local inflammation is induced by the effects of pathogen products mediated through toll like receptors that are expressed by mesenchymal cells, macrophages and epithelial cells. As a result, DCs in the Peyer's patches or lamina propria mature completely after taking up antigen and produce IL-12. After migration to the MLN, these DCs prime gut-homing T_H1 cells, which produce IFN-γ and cause further inflammation. The result of the interaction between intestinal contents, unique anatomical features, and immune and non-immune cells is an environment that favors the induction of IgA antibodies and regulatory-T-cell-dependent tolerance. This ensures that a homeostatic balance is maintained between the intestinal immune system and its antigen load, retaining the ability to recognize both dangerous and harmless antigens as foreign, and preserving the integrity of the intestinal mucosa. Inappropriate immune response to food and commensal bacteria may result in inflammatory response pattern as encountered in IBD.

4 Oral tolerance

The ability of the mucosal immune system to distinguish between harmful and harmless antigens is essential for mounting protective immune responses and preventing the induction of mucosal pathology. One of the mechanisms inhibiting a reactive immune response is the oral tolerance induction. Oral tolerance is defined as the induction of a state of systemic immune unresponsiveness to orally administrated antigen upon subsequent antigen challenge. This mechanism presumably prevents the development of an immune reaction or allergy against intestinal intraluminal antigens. However, the inductive site of oral tolerance and the type of antigen-presenting cells generating the tolerogenic immune response are not yet defined. T cells appear to be the major target of tolerance, and the reduction of antibody responses after antigen feeding are due to a reduction in T helper activity rather than to direct tolerization of B cells.

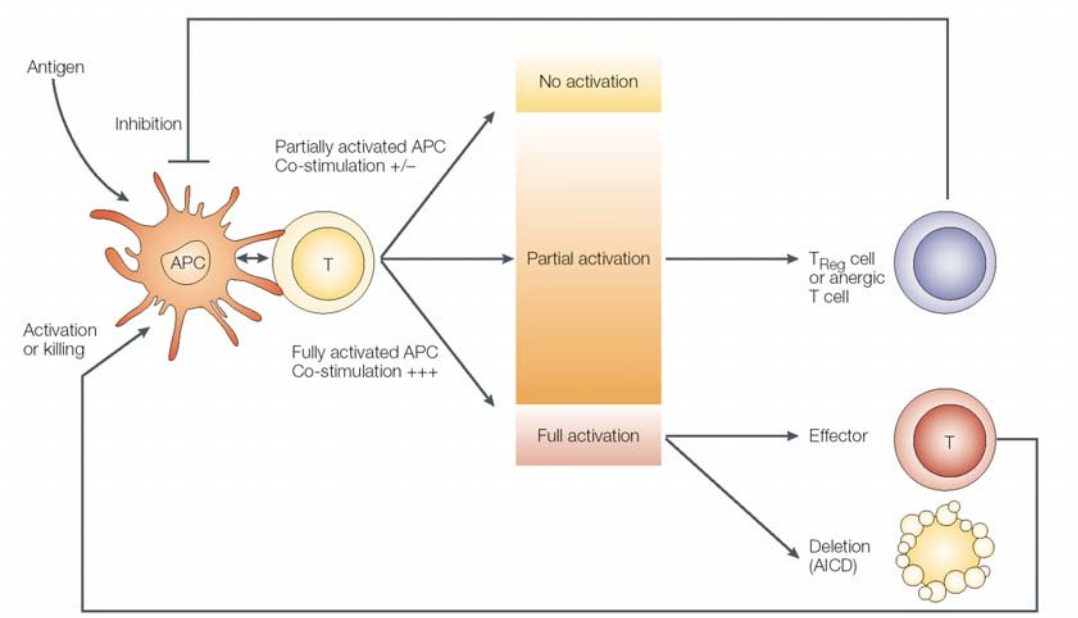


Figure 6: Antigen presentation and tolerance induction. Antigen has to be taken up by APCs. The activation state of the APC has a crucial role in determining the outcome of the ensuing APC-T cell interaction. The absence of an antigen-specific T cell is tolerance in its purest sense. At the other end of the spectrum, a fully activated APC induces T cell activation. Some activated lymphocytes might then undergo activation-induced-cell-death (AICD) and deletion by apoptosis. The intermediate zone between no activation and full cellular activation is connected to T_{reg} cells. Partial activation can generate T cells that are anergic and that have properties of T_{reg} cells, including the ability to render APCs 'tolerogenic' (adapted from Herrath & Harrision, 2003).

The major mechanisms of tolerance induction are clonal deletion, clonal anergy and the induction of suppressor cells (Weiner, 1997; Weiner, 2001). IL-12, a T_H1-directing cytokine, may be the key regulatory cytokine for these various pathways in the mucosal immune response. Factors that suppress IL-12 production by antigen presenting cells result in suppressor or regulatory T cells producing TGF-β and possibly IL-4 and IL-10, while factors that induce IL-12 production result in T cells producing the pro-inflammatory cytokine IFN-γ (Chen et al., 1997; Kelsall et al., 1994; Marth et al., 1996). The nature and localization of the antigen presenting cells responsible for tolerogenic presentation of fed antigens are unclear, but T cell activation and/or deletion can be rapidly observed in the Peyer's patches of antigen fed mice (Chen et al., 1996). Oral tolerance induction is also a way to protect the organism against autoimmune reactions against self antigens and thus to prevent the development of autoimmune disease. Nevertheless, further studies are necessary to characterize the pathways of tolerance induction in humans and mice before using this approach as a therapeutic tool against hyperresponsiveness of the mucosal immune system.

5 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory condition of the gastrointestinal tract that is manifested in two usually distinct but sometimes overlapping clinical entities, ulcerative colitis (UC) and Crohn's disease (CD). IBD leads to long-term and sometimes irreversible impairment of the gastrointestinal structure and function (summarized in Fig. 7).

5.1 Etiology and pathophysiology of IBD

IBD is thought to result from inappropriate and ongoing activation of the mucosal immune system driven by the presence of the normal luminal flora. This aberrant response is most likely facilitated by genetic prepositions and defects in both the barrier function of the intestinal epithelium and the mucosal immune system.

5.1.1 Genetic factors

It has been shown in a variety of different mouse models that genetic defects can lead to the development of spontaneous mucosal inflammation (Tab. 2), demonstrating that entirely different genetic abnormalities can lead to similar clinical features of intestinal inflammation. An example for the genetic predisposition to IBD is represented by the *nod2/card15* gene. About 20 % of patients with Crohn's disease have mutations in this gene, which is involved in the regulation of host responses to bacteria (Hampe et al., 2001). Recently, common structural and functional features between human and mouse NOD2 have been identified (Iwanaga et al., 2003). This should allow the development of relevant animal models to evaluate the role of NOD2 in chronic inflammatory disorders.

Additionally, the host genetic background determines the susceptibility of intestinal inflammation, even when this is due to a major genetic defect. For example, some inbred mouse strains with IL-10 deficiency are highly susceptible to colitis, while others are resistant (Mahler & Leiter, 2002). These differences in disease susceptibility and resistance among mouse strains offer the opportunity to identify murine genes which

Figure 7: Characteristics of inflammatory bowel disease.

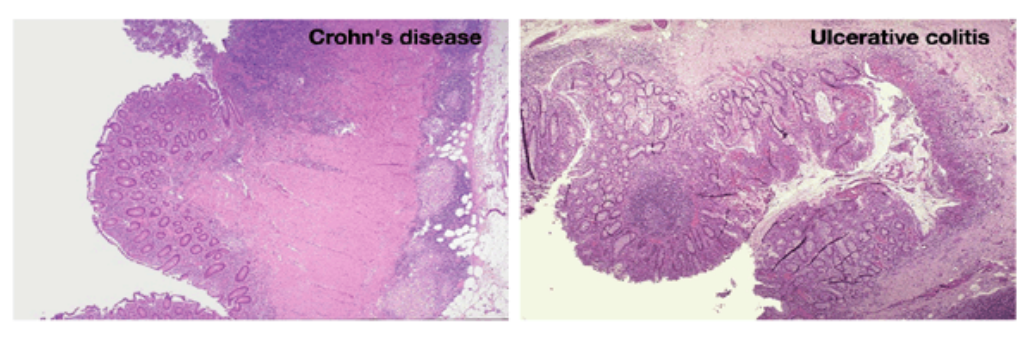
What is inflammatory bowel disease?

General definition
Inflammatory bowel disease (IBD) is a chronic relapsing idiopathic inflammation of the gastrointestinal tract. The two main forms of IBD — Crohn's disease and ulcerative colitis — have many similarities, but there are also several clinical and pathological differences. In a small minority of cases that involve only the colon, they are indistinguishable and categorized as 'indeterminate colitis'.

Epidemiology
Both Crohn's disease and ulcerative colitis have a prevalence range of 10–200 cases per 100,000 individuals in North America and Europe. Disease incidence is the highest in developed, urbanized countries. The incidence of Crohn's disease has increased during the past four decades, whereas no clear trend is identifiable for ulcerative colitis.

Areas of involvement
Crohn's disease. Any part of the gastrointestinal tract can be affected, but most commonly, the terminal ileum, cecum, peri-anal area and colon. It is characterized by the presence of segments of normal bowel between affected regions, known as 'skip' lesions. The intersection of linear ulcers with islands of normal or oedematous mucosa might produce a 'cobblestone' appearance.
Ulcerative colitis. The inflammatory process invariably involves the rectum and extends proximally in a continuous fashion, yet remains restricted to the colon. Sometimes, it is limited to the rectum as 'ulcerative proctitis'.

Histology
Crohn's disease. A transmural (affecting all layers of the bowel wall), dense infiltration of lymphocytes and macrophages; presence of granulomas in up to 60% of patients; fissuring ulceration and submucosal fibrosis (see left-hand figure).
Ulcerative colitis. Inflammation affects superficial (mucosal) layers with infiltration of lymphocytes and granulocytes and loss of goblet cells. Presence of ulcerations and crypt abscesses (see right-hand figure).



(Left). Photomicrograph of a histological specimen taken from a patient with Crohn's disease. Inflammation can be seen to involve the full thickness of the wall from the mucosa to the serosa. Granulomas are seen towards the serosal surface.
Right). Ulcerative colitis is shown microscopically here at low-power magnification to show mucosal inflammation and erosion. High-power magnification might show the presence of acute inflammatory cells in crypts, known as 'crypt abscesses'. Images courtesy of Edward C. Klatt at Florida State University College of Medicine.

Clinical features and complications
Crohn's disease. Diarrhea, pain, narrowing of the gut lumen leading to strictures and bowel obstruction, abscess formation, and fistulization to skin and internal organs.
Ulcerative colitis. Severe diarrhea, blood loss and progressive loss of peristaltic function leading to rigid colonic tube. In severe cases, this can lead to 'toxic megacolon' and perforation.
Extra-intestinal inflammatory manifestations in joints, eyes, skin, mouth and liver can occur in both forms of IBD. Increased risk for colon carcinoma in longstanding IBD — in particular, ulcerative colitis.

Current treatments
Crohn's disease. 5-ASA compounds, corticosteroids, azathioprine/6-MP, methotrexate, antibodies specific for tumour-necrosis factor (TNF) and surgical removal of obstructing segments.
Ulcerative colitis. 5-ASA compounds, corticosteroids, azathioprine/6-MP, intravenous cyclosporin and colectomy.

Bouma & Strober, 2003

play a important role in disease onset and progression, and might therefore contribute to the identification of genes necessary for the occurrence of disease in humans.

5.1.2 Dysregulation of the mucosal immune system

Besides the genetic predisposition the interaction between the intestinal microflora and the mucosal immune system plays a key role in the development of IBD (paragraph 2.2). The immunological nature of the disease arises from the observation that IBD is characterized by massive cellular infiltrates and is associated with abnormalities of the immune system that include inappropriate production of antibodies and T cell dysfunction. This concept has been clarified by studies in human patients with CD. Lamina propria cells showed an overproduction of cytokines indicative of a typical helper T_H1 response, namely increased production of IL-12 by LP macrophages and increased production of INF-γ by LP T cells (Fiocchi, 1998; Fuss et al., 1996; Monteleone et al., 1997). In addition, LPL from patient with UC developed a cytokine profile resembling a T_H2 response. More precisely, LPL from UC patients do not produce high levels of the major T_H2 cytokine IL-4, but they produce increased amounts of another T_H2 cytokine IL-5. These studies provide circumstantial evidence that the two major forms of IBD in humans are a consequence of dysregulated or excessive T_H1 (CD) or T_H2 (UC) responses. There is considerable evidence that IBD patients have inappropriate T cell responses to their own intestinal flora, either because of dysfunction in the primary or secondary mechanisms that normally drive and regulate such responses or due to some dysfunction in the intestinal epithelial cell barrier that leads to inappropriate penetration of microbial agents (Duchmann et al., 1995; Soderholm et al., 1999; Probert et al., 1996). In effect, patients with IBD have a failure in the maintenance of oral tolerance including down-regulation of responses to harmless luminal antigens like commensals or food, while allowing effector cell responses to mucosal pathogens.

5.2 Animal models of mucosal inflammation

In the recent years the status of IBDs as canonical autoimmune diseases has risen steadily with recognition that these diseases are abnormalities in mucosal responses to normally harmless antigens derived from the mucosal microflora and therefore responses to antigens that, by their proximity and persistence, are equivalent to self-

antigens (Strober et al., 2002). This new paradigm is consistent with the fact that multiple murine models (Tab. 2) of mucosal inflammation exist which affect the immunological balance and can lead to loss of tolerance to mucosal antigens and thus inflammation centered in the gastrointestinal tract. All models have their individual capacities to provide insights into IBD pathogenesis. It emerges that murine models of mucosal inflammation will allow to define and understand the immunology of IBD in all its complexity and to find unexpected ways to treat these diseases.

Most models for IBD are based on dysregulated effector functions or changes in the regulatory processes. The great majority of existing models are T_H1 based models. One explanation relates to the fact, that in most if not all models the inflammation is driven by antigens of the normal mucosal microflora like LPS, CpG-motive or superantigens (Strober et al., 2002). The mitogen-lymphocyte interaction predominantly induces an IL-12 dependent T_H1 immune response. Moreover, the regulatory effect of the cytokines TGF-β and IL-10 is mainly based on T_H1 responses. Also the nature of the antigen is an important factor for the developing immune response. It was shown that two classical skin sensitizing agents induced different forms of inflammation in mice. Trinitrobenzene sulfonic acid induces a T_H1 response in SJL/J mice and oxazalone promotes a non classical T_H2 response in these mice (Boirivant et al., 1998).

5.2.1 Chemically induced models

This group of models requires administration of a chemical agent for the induction of colitis. Examples include trinitrobenzene sulfonic acid (TBNS) (Morris et al., 1989), dextran sodium sulfate (DSS) (Okayasi et al., 1990), and oxazolone (Boirivant et al., 1998). Chemically induced models are useful for studying biochemical pathways of inflammation or for performing antigen-specific studies, such as in the case of hapten-induced gut inflammation (TBNS). In addition, these models are particularly valuable in the dissection of specific aspects or events on the overall background of intestinal inflammation. For example, DSS-induced colitis is characterized by epithelial disruption resulting in luminal bacterial translocation and subsequent infiltration of neutrophils and other acute phase immune cells. However, although these events might be important in initiating gut inflammation, DSS colitis can be induced in the absence of lymphocytes (Dielman et al., 1998), and does not represent the chronic phases of disease. Therefore, DSS colitis can be considered as an appropriate animal model to investigate epithelial

response to injury, neutrophil infiltration or other aspects of the acute phase of colitis pathogenesis, but this not adequately addresses those events occurring during the chronic phase of gut inflammation. Nevertheless, important informations have been derived using these experimental systems, in particular studies regarding the pathogenic role of specific cytokines in experimental colitis (Neurath et al., 1995).

Table 2: Commonly used mouse models of intestinal inflammation and IBD

Animal model	Disease type	Reference
Chemically induced		
TNBS	Colitis, acute, chronic, transmural, T_H1	Morris et al., 1989
DSS	Colitis, superficial, Th1 (acute), T_H1/T_H2 (chronic)	Okayasi et al., 1990
Oxazolone	Colitis, T_H2	Boirivant et al., 1998
Immunological		
$CD4^+CD45RB^{high}$ SCID transfer	Colitis, chronic transmural, T_H1	Morrissey et al., 1993
Tgε bone marrow chimera	Colitis, T_H1	Hollander et al., 1995
Genetic		
IL-10 KO	Colitis, acute, chronic, transmural, T_H1 (early) / T_H2 (late)	Kuhn et al., 1993
$TNF^{\Delta ARE}$	Ileocolitis, chronic, T_H1, transmural, granulomatous	Kontoyiannis et al., 1999
Spontanous		
C3H-HeJBir	Cecitis, superficial, acute resolving, T_H1	Sundberg et al., 1994
SAMP1/Yit	Ileitis, chronic, transmural, granulomatous, T_H1	Matsumoto et al., 1998
SAMP1/YitFc	Perianal disease, early onset of disease	Rivera-Nieves et al., 2003

Abbreviations: DSS, dextran sodium sulphate; SCID, severe combined immunodeficient, TNBS, trinitrobenzene sulfonic acid (adapted from Pizzaro et al., 2003).

5.2.2 Immunological models

Immunological induced models for IBD are mainly based on adoptive transfer of T cells or bone marrow precursors, which are introduced into immunodeficient recipient mice. Classical examples are the $CD4^+CD45RB^{high}$ (Morrissey et al., 1993), the bone marrow chimera (Hollander et al., 1995) and the $CD4^+CD25^-$ (Mottet et al., 2003) transfer model. Furthermore, the transfer of hsp60-reactive $CD8^+$ T cells is also sufficient to induce intestinal inflammation, primarily in the small intestine (Steinhoff et al., 1999). Studies in these models have elucidated the role of pathogenic and regulatory T cells in controlling mucosal immunity and intestinal inflammation and offer strong evidence that T_H1 polarization plays a key role in CD pathogenesis (Powrie, 1995). However, the profound immune abnormalities in the recipient mice with totally absence of T and B cells probably make these models unsuitable for investigating the innate factor causing human CD.

5.2.3 Genetic models

Transgenic and knock-out methodologies have revolutionized the field of animal models for to IBD. With the exception of a few transgenic models (e.g. E-cadherin transgenic mice), the majority of these genetic mouse models are gene knock-outs. Examples include IL-2 (Sadlack et al., 1993), TCR αβ (Mombaerts et al., 1993), IL-10 (Kuhn et al., 1993), and Gi1-a (Rudolph et al., 1995) knock-out models. Genetic models greatly contribute to our understanding of the role of key immune-related molecules in the pathogenesis of chronic intestinal inflammation. Collectively, these models have clearly established the requirement for strict regulation of the mucosal immune response and have allowed the identification of key components involved in gut immune regulation. However, it is unlikely that the imposed genetic mutations represent the underlying defect in human IBD, limiting the utility of these models for understanding causative factors in both ulcerative colitis and Crohn's disease.

5.2.4 Spontaneous models

Spontaneous models represent one of the most attractive tools for studying intestinal inflammation, because similar to human disease inflammation occurs without any apparent exogenous manipulation. For example, the C3H/HeJBir murine model of colitis is characterized by spontaneous and chronic focal inflammation localized to the right colon and cecal region. Colitis occurs in young mice and tends to resolve with age

without recurrence (Sundberg et al., 1994). In these spontaneous models it is possible to study the role of immunobacterial interactions for the induction and chronicity of intestinal inflammation.

5.3 Balance between inflammation and regulation

As a rule, chronic colitis only occurs when the appropriate microbial agent stimulates mucosal immune responses in genetically susceptible hosts. In genetically resistant hosts, intestinal homeostasis is maintained by exclusion of luminal microbial constituents by an intact mucosal barrier and a net suppressive tone of the mucosal immune system, leading to immunologic tolerance to autologous bacteria (Fig. 8).

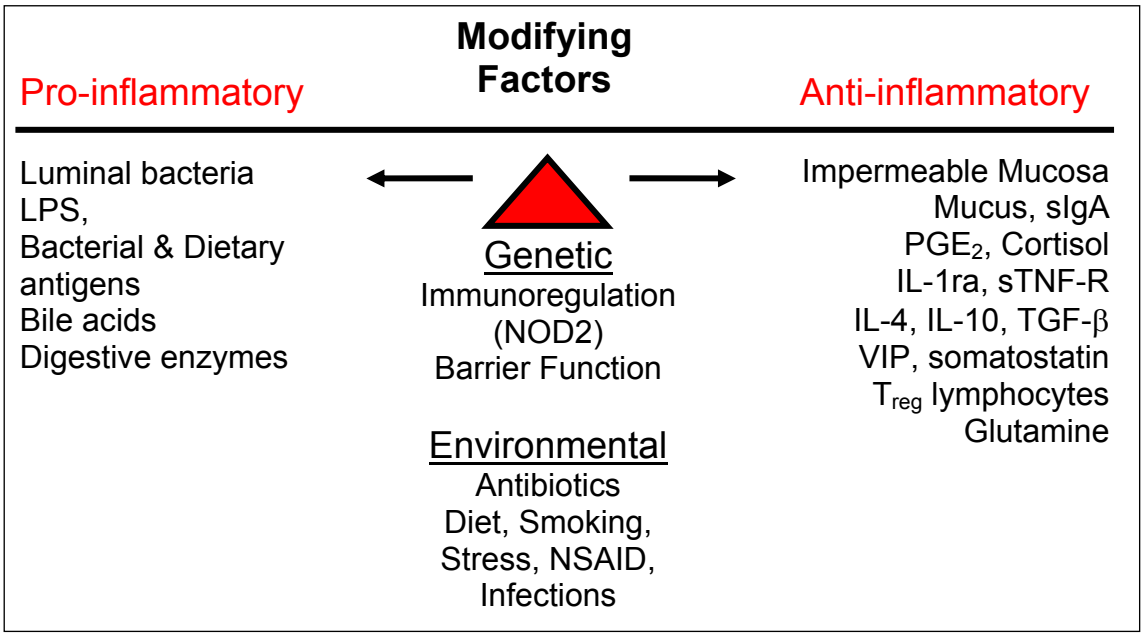


Figure 8: Balance between luminal pro-inflammatory factors and mucosal protective mechanisms. The genetically determined immune response to bacterial products and epithelial barrier functions can influence host susceptibility to chronic inflammation, while environmental factors can influence initial onset and spontaneous reactivation of inflammation (adapted from Sartor, 1997).

This delicate balance can be perturbed by genetic defects of immune regulation and barrier functions as well as by environmental triggers which can initiate a reactive disease. A variety of genetic alterations can adversely affect immune regulation and barrier functions, leading to phenotypically similar chronic intestinal inflammation. The luminal microbial environment is incredible complex, with over 300 - 500 individual

bacteria strains. This complexity may be even further augment if different genetic subsets of patients respond to different dominant bacterial stimuli, as suggested by animal models. However, recent investigations in animal models provide hope that selective elimination of dominant bacterial antigenic stimuli by specific antibiotics or even competition with probiotic strains can complement new selective immunotherapies against chronic intestinal inflammation.

CHAPTER II

Induction of chronic mucosal inflammation in mice by enterocyte-specific $CD4^+$ T cells

Induction of chronic mucosal inflammation in mice by enterocyte-specific $CD4^+$ T cells

1 Background

Several studies have suggested that chronic inflammatory bowel disease might be a consequence of antigen specific recognition of appropriate T cells which expand and induce immunopathology. Since the intestinal immune system has to discriminate between harmless antigens derived from nutrients or bacterial flora on the one hand and harmful antigens derived from pathogens on the other hand, induction and maintenance of mucosal tolerance is of indispensable importance to avoid inappropriate immune responses in the gut (Nagler-Anderson, 2001). Central tolerance induction takes place in the thymus, where clonal deletion of potentially auto-reactive T cells occurs (Kisielow et al., 1988; von Boehmer et al., 2003). However, in few cases auto-reactive T cells escape thymic deletion, but these T cells are usually rendered anergic due to the absence of costimulatory signals on their target tissue in the periphery (Melamed & Frieman, 1993). Because of the huge variety of antigens and the large number of lymphoid cells in the intestine, minor dysfunctions of mucosal immune homeostasis may result in intestinal immune responses resulting in inflammation and chronic disease (Monteleone et al., 2002). Therefore, additional tolerance mechanisms must exist to tightly control inappropriate immune responses. It has been shown that maintenance of tolerance in the gut can be mediated by natural occurring $CD4^+CD25^+$ or $CD4^+CD45RB^{low}$ regulatory T cells which suppress uncontrolled immune responses most likely towards luminal antigens (Sakaguchi, 2003). This is achieved by secretion of regulatory cytokines like interleukin (IL)-10 and transforming growth factor (TGF)-β (Powrie et al., 1994; De Winter et al., 2002). Regulatory T cells suppress intestinal pathology mediated by T cells, but until now it remains unclear how they elicit their effector function *in vivo* (Maloy & Powrie, 2001).

2 Aims of the study

To analyze the immunological and molecular mechanisms of autoantigen-specific $CD4^+$ T cell response in chronic mucosal inflammation a transgenic mouse expressing hemagglutinin A/PR8/34 (HA) under the control of the gut-specific villin promotor in enterocytes of the intestinal epithelium was generated (Templin et al., submitted). To establish an autoimmune environment these VILLIN-HA mice were crossed with TCR-HA mice expressing a transgenic T cell receptor specific for the MHC class II restricted peptide HA110-120 (Kirberg et al., 1994). Concomitant expression of HA and a MHC class II-restricted T cell receptor specific for HA resulted in an autoimmune mediated chronic intestinal inflammation. The mild form of mucosal inflammation suggested the induction of peripheral tolerance mechanisms. To study these mechanisms in more detail, extensive immunological characterization of self-reactive T cells should be performed including:

- Isolation of peripheral self-reactive enterocyte specific T cells from VILLIN-HA x TCR-HA double transgenic mice and TCR-HA control mice to characterize the proliferative capacity and their activation status.

- Morphological evaluation of the intestine from VILLIIN-HA x TCR-HA double transgenic to define the degree of intestinal disease.

- Isolation of auto-reactive intestinal T cell from VILLIN-HA x TCR-HA double transgenic mice to characterize their proliferative capacity.

- Stimulation of the auto-reactive intestinal T cells and determination of the cytokine secretion profile in autoimmune mediated chronic intestinal inflammation.

- Gene expression profiling of self-reactive intestinal T cell from double transgenic VILLIN-HA x TCR-HA mice and TCR-HA control mice.

- Studies on the impact of naturally occurring T_{reg} cells on the outcome of disease in an adoptive transfer system based on VILLIN-HA single transgenic mice.

3 Results

3.1 Enterocyte-specific CD4$^+$ T cells are present in the periphery of VILLIN-HA x TCR-HA mice and proliferate upon antigen stimulation

The prerequisite for the development of autoimmunity is inefficient thymic deletion of the T cell recognizing self-antigens resulting in the presence of auto-reactive T cells in the periphery. Therefore, a key question to answer was whether HA-specific (6.5$^+$) CD4$^+$ T cells mature in the thymus of VILLIN-HA x TCR-HA double transgenic mice and thus can be found in peripheral lymphoid organs. To this end, T cells from spleen and MLN of VILLIN-HA x TCR-HA and TCR-HA control mice were isolated and analyzed by flow cytometry for the expression of the transgenic T cell receptor (Fig. 1).

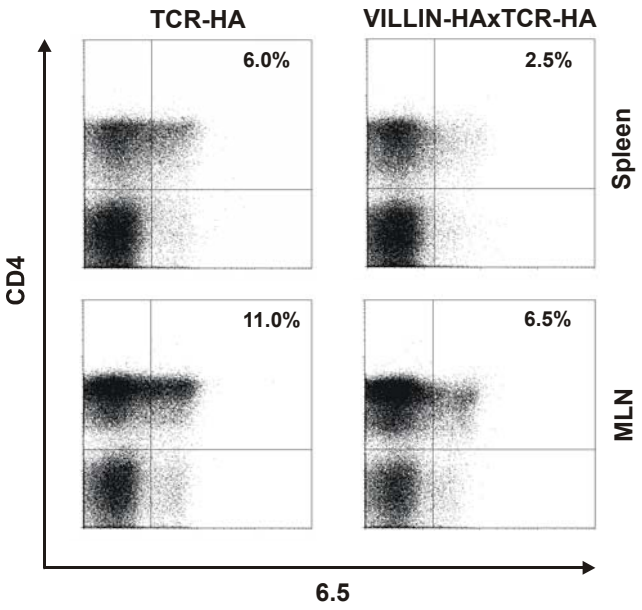


Figure 1: HA-specific CD4$^+$ T cells in the periphery of VILLIN-HA x TCR-HA mice. VILLIN-HA x TCR-HA and TCR-HA control mice were sacrificed, spleen and MLN cells were isolated and stained for 6.5 and CD4 expression to measure the percentage of transgenic T cells in the different compartments.

Indeed, HA-specific 6.5$^+$CD4$^+$ transgenic T cells could be detected in the peripheral lymphatic organs. In the spleen of VILLIN-HA x TCR-HA 2.5 % of the CD4$^+$ T cells carry the transgenic T cell receptor compared to 6.5 % in the MLN of the double transgenic mice. Transgenic cell numbers were decreased in comparison to TCR-HA control mice, suggesting that thymic HA expression in the VILLIN-HA x TCR-HA transgenic mice leads to thymic deletion of a proportion of 6.5$^+$CD4$^+$ T cells in double

transgenic mice. It has been shown previously that expression of HA under control of the Ig-κ promoter by hematopoetic cells resulting in permanent antigen expression both in thymus and in the periphery leads to tolerance rather than inflammation (Buer et al., 1998). Therefore, it was reasonable to analyze whether mature $6.5^{+}CD4^{+}$ T cells found in the peripheral lymphoid organs of VILLIN-HA x TCR-HA double transgenic mice are functional with respect to their proliferative capacity upon antigen encounter. To this end, T cells from spleen and MLN of VILLIN-HA x TCR-HA and TCR-HA control mice were isolated and stimulated *in vitro* with the specific HA peptide. Flow cytometry analysis and normalization of cell numbers ensured that the same percentage of $6.5^{+}CD4^{+}$ T cells from double and single transgenic mice were used for the experiments. Apparently, enterocyte-specific expression of HA did not lead to tolerance induction in peripheral compartments, as demonstrated in Fig. 2. No differences in their capacity to proliferate upon stimulation with their cognate peptide could be observed between T cells isolated from double-transgenic VILLIN-HA x TCR-HA and TCR-HA mice that served as controls (Fig. 2).

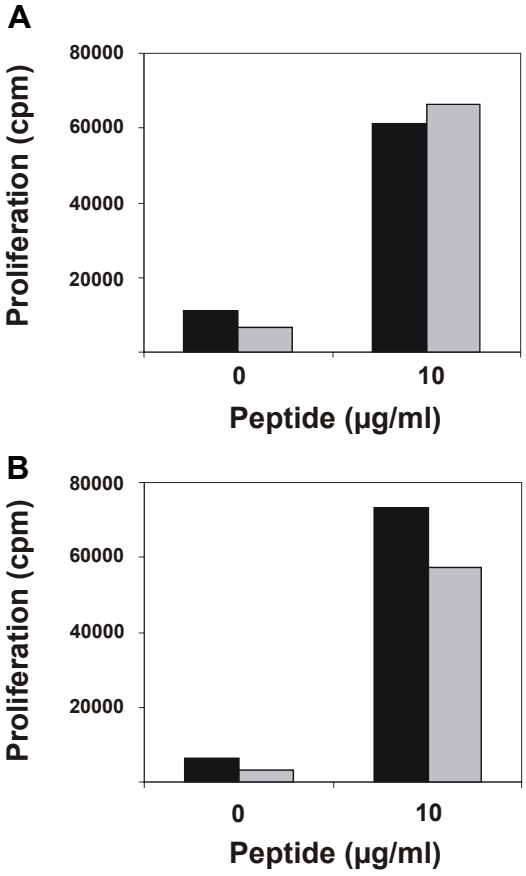


Figure 2:. Proliferative capacity of HA-specific $CD4^{+}$ T cells from spleen and MLN of VILLIN-HA x TCR-HA mice and TCR-HA control. Splenic and lymph node cells from VILLIN-HA x TCR-HA and TCR-HA control mice were isolated and identical numbers of antigen specific $6.5^{+}CD4^{+}$ T cells from spleen **(A)** and MLN **(B)** were used for *in vitro* proliferation assays in presence or absence of 10µg/ml HA-peptide. Proliferation was measured by ^{3}[H]-thymidine incorporation. Black bars represent proliferation of T cells from TCR-HA control mice and grey bars proliferation of VILLIN-HA x TCR-HA derived cells.

3.2 HA-specific CD4$^+$ T lymphocytes from VILLIN-HA x TCR-HA mice have an activated phenotype

To characterize peripheral 6.5$^+$CD4$^+$ T cells from VILLIN-HA x TCR-HA mice in more detail, these cells were analyzed for the expression of the activation and memory markers CD69, CD25, CD45RB and CD62L by flow cytometry (Fig. 3). Comparing the expression patterns of VILLIN-HA x TCR-HA and TCR-HA mice revealed that in the spleen and the MLN of VILLIN-HA x TCR-HA mice the expression of CD45RB was drastically decreased. In the spleen of VILLIN-HA x TCR-HA there is an increased amount of CD69-positive T cells compared to TCR-HA mice, which was even more prominent in the MLN of VILLIN-HA x TCR-HA mice. Compared to TCR-HA mice the percentage of CD25$^+$ T cells was increased in spleen and MLN of double transgenic mice.

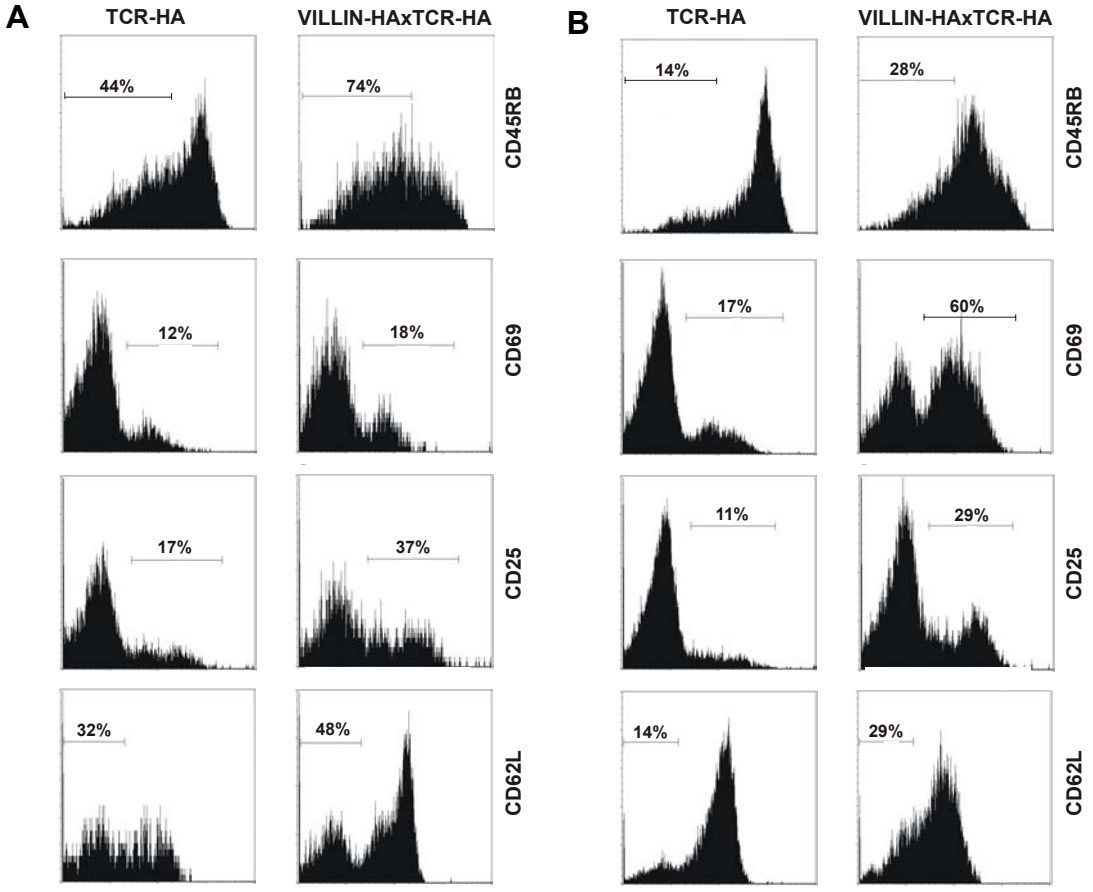


Figure 3: Activation pattern of HA-specific CD4$^+$ T cells from double transgenic VILLIN-HA x TCR-HA mice compared to TCR-HA mice. 6.5$^+$CD4$^+$ T cells were isolated from spleen and MLN of VILLIN-HA x TCR-HA and TCR-HA mice, respectively. Lymphocytes were stained with antibodies 6.5 and CD4 as well as with CD25, CD45RB, CD62L, CD69 antibodies. Cells were gated for 6.5 and CD4 expression on splenocytes **(A)** and MLN **(B)** and analyzed regarding the expression of the different activation/memory markers by FACS.

Additionally the expression of the surface marker CD62L (L-selectin) was determined which is highly expressed on naïve T lymphocytes, but is down-regulated upon activation of T cells. Again, the percentage of $6.5^{+}CD4^{+}$ T cells isolated from spleen and MLN that not expressing CD62L was significantly increased in double transgenic mice compared to control TCR-HA mice. Comprising, peripheral HA-specific $CD4^{+}$ T cells from VILLIN-HA x TCR-HA transgenic mice showed an activated phenotype.

3.3 Morphological evaluation of the intestine

As mature $6.5^{+}CD4^{+}$ T cells of VILLIN-HA x TCR-HA transgenic mice can be found in the periphery of these mice and these autoantigen-specific T cells are able to proliferate upon stimulation with their corresponding antigen and have an activated phenotype, the ability of the $6.5^{+}CD4^{+}$ T cells to infiltrate the intestine, i.e. the compartment where the antigen is located, was investigated. The small intestine of VILLIN-HA x TCR-HA and TCR-HA control mice was isolated, fixed in formalin, embedded in paraffin and sectioned. Sections were H&E stained and immunohistochemistry was performed to identify T cells in the intestine. The evaluation revealed increased numbers of IEL and LPL with moderate lymph edema in the intestine. However, tissue damage to the epithelial cell layer could not be observed, suggesting a mild form of mucosal inflammation (Fig 4).

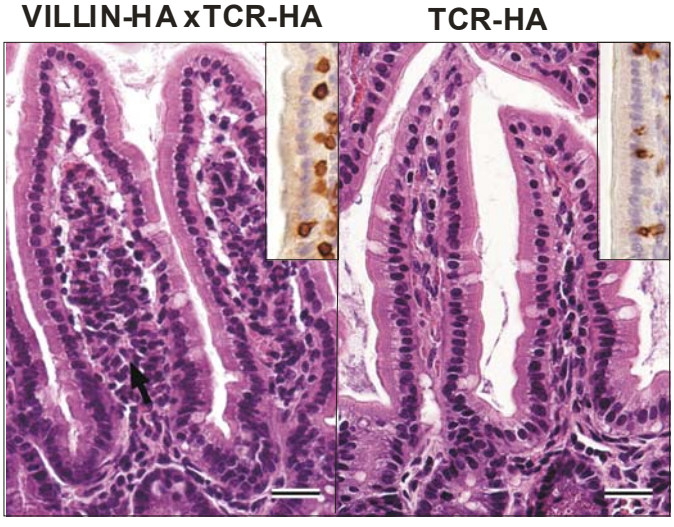


Figure 4: Mild enterocolitis in VILLIN-HA x TCR-HA double transgenic mice characterized by infiltration of lymphocytes into the lamina propria and intestinal epithelium. Intestinal villi are distended (arrow) by increased numbers of lymphocytes (left panel) when compared to intestinal villi of TCR-HA transgenic mice (right panel). Similarly, the number of intraepithelial lymphocytes (IEL) is increased (insets). Insets show α-CD3 immunohistochemistry on paraffin-embedded tissues. ABC method with diaminobenzidine as substrate (brown color) and hematoxylin counterstain (blue nuclei). Ileum, H&E stain, scale bar: 80 µm.

3.4 Functional and molecular characterization of auto-reactive intestinal CD4$^+$ T cells

It is well established, that mucosal lymphocytes are relatively unresponsive to T cell receptor dependent stimulation compared to peripheral blood lymphocytes. To evaluate the responsiveness of auto-reactive 6.5$^+$CD4$^+$ T cells isolated from the small intestine to antigenic stimulation, IEL and LPL from VILLIN-HA x TCR-HA as well as TCR-HA control mice were isolated and stimulated *in vitro* with the corresponding peptide. Proliferation was measured by ^{3}[H]-thymidine incorporation and culture supernatants were analyzed for several cytokines by cytokine bead array.

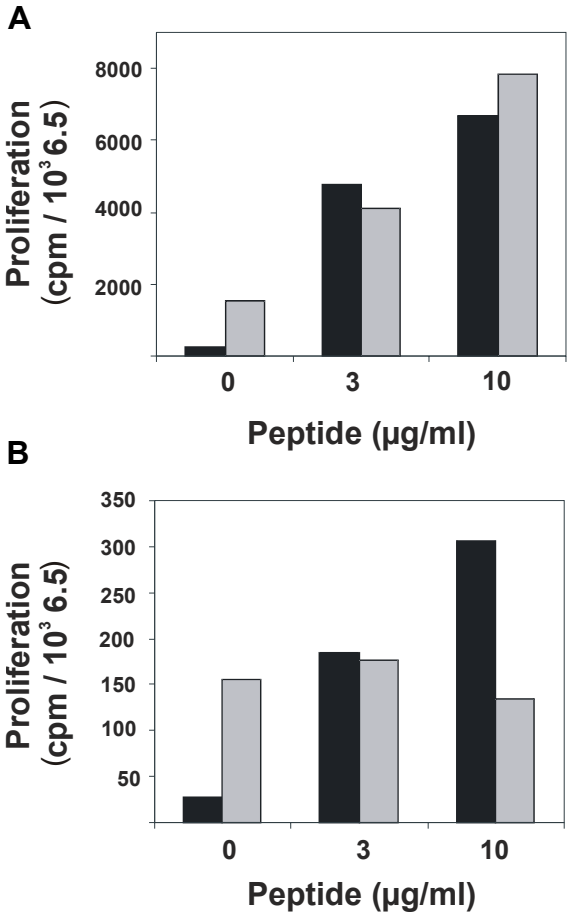


Figure 5: Reduced proliferative capacity of HA-specific IEL from VILLIN-HA x TCR-HA mice. LPL **(A)** and IEL **(B)** were isolated from VILLIN-HA x TCR-HA and TCR-HA control mice and stimulated *in vitro* with the corresponding HA-peptide. Thymidine incorporation was referred to cpm per thousand 6.5$^+$CD4$^+$ T cells. Proliferation of responder cells from TCR-HA mice is depicted in black bars and in grey bars for VILLIN-HA x TCR-HA derived cells.

LPL from VILLIN-HA x TCR-HA mice as well as LPL from TCR-HA mice proliferate in a dose dependent manner. In contrast, the proliferative capacity of IEL from VILLIN-HA x TCR-HA mice to antigenic stimulation was abrogated with a high background proliferation in comparison to IEL from TCR-HA single transgenic mice (Fig. 5).

Fig. 6 and Fig. 7 show the cytokine secretion profiles of LPL and IEL from VILLIN-HA x TCR-HA and TCR-HA control mice. Antigen-stimulated $6.5^{+}CD4^{+}$ LPL from diseased mice secrete significantly lower amounts of IFN-γ and IL-2 upon *in vitro* stimulation, both of which are cytokines normally involved in the induction of gut inflammation (Fiocchi, 1998). Additionally, IEL from VILLIN-HA x TCR-HA mice secreted lower levels of IFN-γ compared to control TCR-HA mice. In contrast, in LPL and IEL from double transgenic mice the basal level secretion of TNF-α, MCP-1 and IL-6, which are also discussed as important mediators in the context of IBD, was considerably increased.

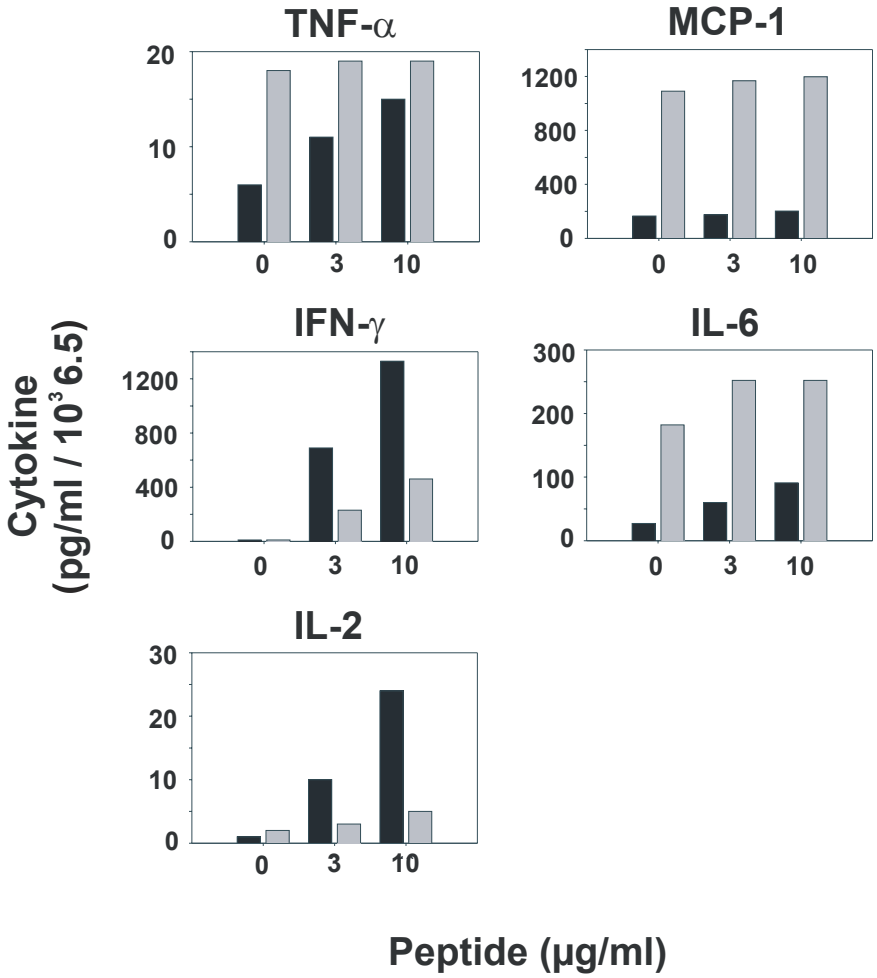


Figure 6: Cytokine profile of HA-specific LPL from the inflamed intestine. LPL from TCR-HA (black bars) and VILLIN-HA x TCR-HA (grey bars) mice were stimulated *in vitro* with the HA110-120 peptide. Culture supernatants were analyzed for several cytokines using the cytokine bead array from BD. Cytokine quantities are depicted as pg/ml per thousand $6.5^{+}CD4^{+}$ intestinal T cells.

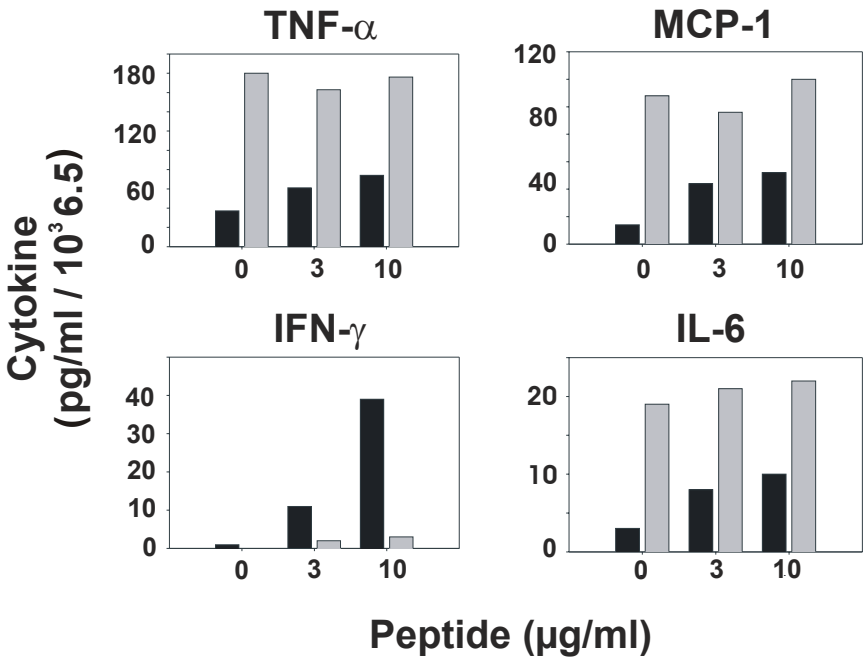


Figure 7: Cytokine profile of HA-specific IEL from the inflamed intestine. IEL from TCR-HA (black bars) and VILLIN-HA x TCR-HA (grey bars) mice were stimulated *in vitro* with the HA110-120 peptide. Culture supernatants were analyzed for several cytokines using the cytokine bead array from BD. Cytokine quantities are depicted as pg/ml per thousand $6.5^{+}CD4^{+}$ intestinal T cells.

Together, in contrast to LPL, IEL from VILLIN-HA x TCR-HA mice are unresponsive to antigenic stimulation and moreover, cytokine pattern of LPL and IEL from diseased mice clearly differs from that of TCR-HA control mice. These cytokine data clearly indicate that there is an unbalance in the regulatory environment of the intestine. On the one hand there is a down-regulation of pro-inflammatory cytokines such as IFN-γ and IL-2, on the other hand LPL and IEL secrete increased levels of TNF-α, MCP-1 and IL-6 even without further antigenic stimulation *in vitro*.

3.5 Global gene expression profiling of self-reactive mucosal $CD4^+$ T cells

As enterocyte-specific antigen expression obviously has a strong impact on the function of auto-reactive $CD4^+$ T cells these T cells were extensively characterized by global gene expression profiling. $CD4^+6.5^+$ T cells from the epithelium and the lamina propria of the small intestine of four individual VILLIN-HA x TCR-HA and four TCR-HA mice were isolated by cell sorting. RNA was prepared and subjected to differential gene expression analysis using Affymetrix MG-U74Av2 oligonucleotide arrays. The advantage of this technology is that every gene analyzed is represented by sixteen independent probe pairs which together establish the basis for statistical evaluations of the respective signals. Therefore, only those genes that are reproducibly regulated are included in the analysis. For each gene fulfilling these criteria, the average fold change in expression for 6.5^+CD4^+ IEL and LPL from VILLIN-HA x TCR-HA and TCR-HA mice was calculated and the ratio was depicted on a base-2 logarithmic scale. To get an impression of the basal expression level of analyzed genes in IEL and LPL under normal conditions, an alignment of LPL versus IEL derived 6.5^+CD4^+ T cells from TCR-HA mice was also included. This approach led to a comprehensive overview about the functional gene classes involved in autoimmune-mediated intestinal inflammation, including surface antigens, regulators of transcription and translation, secreted or signaling molecules and genes involved in cell cycle, apoptosis and survival. Six clusters of co-regulated genes (Fig. 8 A-F) were found to be of special interest as they combine genes either specifically up-regulated (A) or down-regulated (B) in both LPL and IEL, exclusively up-regulated (C) or down-regulated (D) in LPL, as well as up-regulated (E) or down-regulated (F) only in IEL due to intestinal inflammation in VILLIN-HA x TCR-HA mice. Noticeable, although the basal level expression of the majority of genes analyzed is similar in LPL and IEL (third column, LPL vs IEL), most of the differentially expressed genes are regulated exclusively in one of these subpopulations of auto-reactive mucosal $CD4^+$ T cells upon intestinal inflammation.

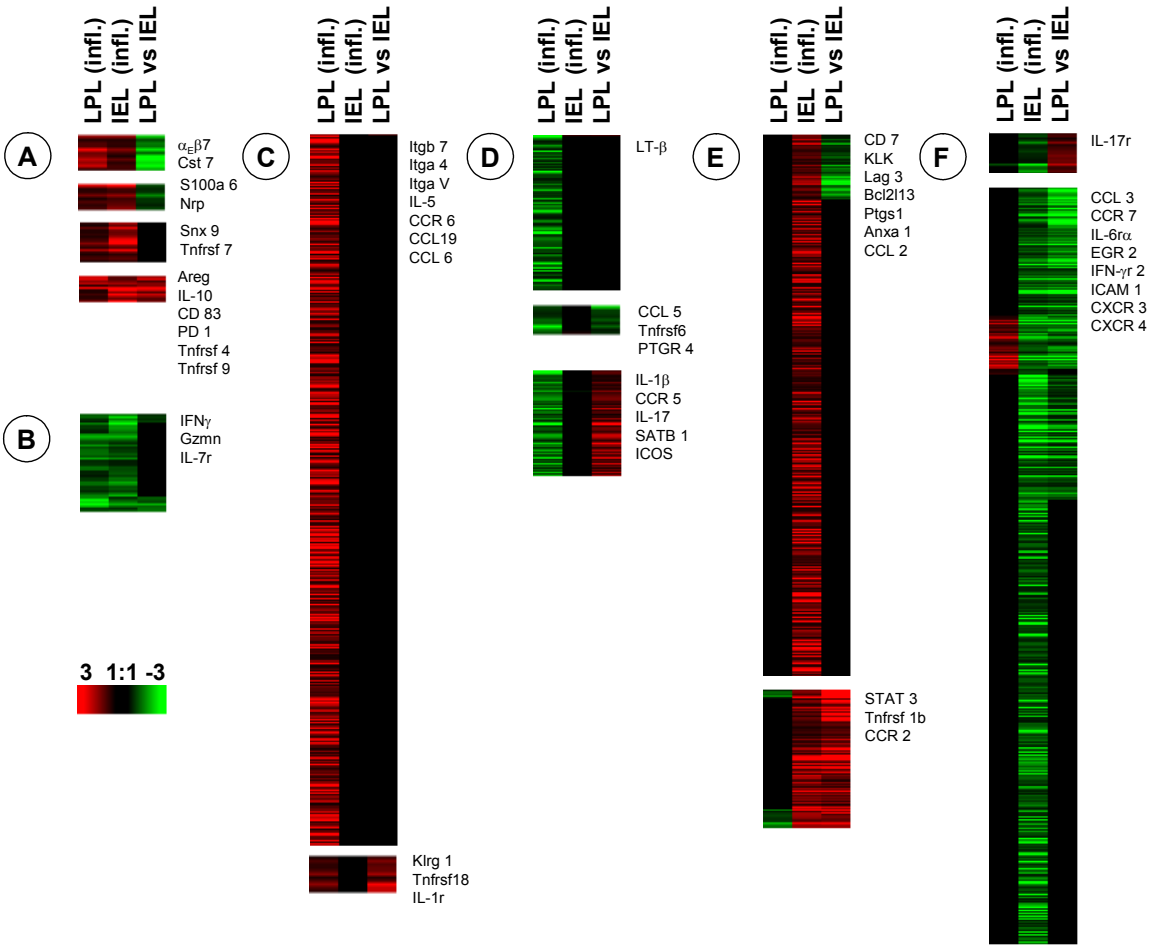


Figure 8: Global gene expression profiling of HA-specific CD4$^+$ T cells. Cluster analysis of genes differentially expressed in 6.5$^+$CD4$^+$ T cells isolated from lamina propria and epithelium of diseased VILLIN-HA x TCR-HA as well as healthy TCR-HA mice. Red indicates induction of gene expression, green indicates repression. As brighter the color as stronger the factor of gene regulation (+3: bright red; -3: bright green). Black indicates no changes. Inclusion into this heat map required at least a 1.5-fold difference in inducible gene expression. LPL (infl.), represents genes differentially expressed in 6.5$^+$CD4$^+$ T cells from the inflamed lamina propria of VILLIN-HA x TCR-HA mice compared to the LPL of healthy TCR-HA donors. IEL (infl.), represents gene differentially expressed in 6.5$^+$CD4$^+$ T cells from the epithelium of VILLIN-HA x TCR-HA mice compared to TCR-HA. LPL versus IEL characterizes basal level expression of genes by LPL compared with the IEL of healthy TCR-HA mice. Cluster A: Genes up-regulated in the LPL and IEL of VILLIN-HA x TCR-HA mice upon gut inflammation. Cluster B: Genes down-regulated in LPL and IEL during inflammation. Cluster C: Genes exclusively expressed by LPL at a higher level than by LPL under healthy conditions. Cluster D: Genes, which are down-regulated in self-reactive LPL CD4$^+$ T cells in the inflamed gut. Cluster E: Genes exclusively up-regulated by IEL from inflamed tissue. Cluster F: Genes down-regulated by IEL from VILLIN-HA x TCR-HA mice.

Among the genes analyzed various integrins significantly up-regulated in LPL and/or IEL from the inflamed tissue were found. $\alpha_E\beta_7$ is highly expressed on both LPL and IEL, whereas Itgb7 and Itga4 and ItgaV are up-regulated only in LPL from the inflamed intestine (Fig.8 A and C). Normally, integrins are involved in lymphocyte homing to the intestinal mucosa and it has been demonstrated that their expression is often enhanced upon intestinal inflammation. Consequently, anti-adhesion molecule treatment in some cases results in attenuated progression of colitis (Elewaut et al., 1998; Sun et al., 2001; Hornquist et al., 1997; Ghosh et al., 2003; Van Assche et al., 2002; Podolsky et al., 1993). Several members of the TNF receptor superfamily (Tnfrsf) were found to be differentially expressed in LPL and IEL from double transgenic mice. Elevated numbers of peripheral T cells in inflammatory bowel disease display Tnfrsf7 and Tnfrsf9 (Raedler et al., 1985; Croft, 2003). In line with these results, Tnfrsf7 and Tnfsrf9 were up-regulated by self-reactive LPL and IEL from VILLIN-HA x TCR-HA transgenic mice (Fig. 8 A). Tnfrsf4 (OX40) shows an ambiguous phenotype of expression. On the one hand Tnfrsf4 is expressed on activated $CD4^+$ T cells and constitutive Tnfrsf4/Tnfrsf4L interaction induces autoimmune-like diseases (Stüber et al., 2000; Murata et al., 2002), on the other hand it is highly expressed on regulatory T cells (McHugh et al., 2002; Walkers et al., 2003). In agree with these observations, Tnfrsf4 expression was also up-regulated in LPL and IEL from diseased VILLIN-HA x TCR-HA mice (Figure 8 A). Tnfrsf18 (GITR) has been shown to play a key role in immunological self-tolerance maintained by $CD4^+CD25^+$ regulatory T cells and was described as a suitable molecular target for preventing or treating autoimmune disease (Shimizu et al., 2002). This molecule was also up-regulated in LPL isolated from diseased tissue of VILLIN-HA x TCR-HA transgenic mice (Fig. 8 C). Besides integrins and Tnfrsf members, another important group of molecules expressed by LPL and IEL and known to be involved in the induction and/or regulation of gut inflammation are cytokines. IL-10 expression was highly up-regulated in LPL and IEL of VILLIN-HA x TCR-HA transgenic mice compared to control mice (Fig. 8 A). T_{reg} cells are known to express high levels of IL-10 and are able to suppress T cell proliferation. It was shown that the transfer of IL-10 transduced T cell in SCID mice, results in prevention of colitis and trials with human IL-10 secreting $CD4^+$ T cells delivered a novel approach to local delivery of immunomodulatory signals to the intestine in IBD (Van Montfrans et al., 2002). In LPL from diseased tissue also IL-5 was significantly up-regulated (Fig. 8 C). It was shown by Fuss et al. (1996) that LPL manifest increased secretion of the T_H2

cytokine IL-5 in ulcerative colitis. Furthermore, other studies figured out that lymphotoxin β (LT-β) is expressed in chronic inflammatory conditions and IL-17 expression in the mucosa and the serum is increased in IBD patients (Agyekum et al., 2003; Nielsen et al., 2003; Fujino et al., 2003). In contrast, in LPL from inflamed gut tissue in the VILLIN-HA x TCR-HA model the expression of LT-β and IL-17 were down-regulated, resembling the regulatory capacity of the intestine to maintain immunological balance (Fig. 8 D). In addition to cytokines, the expression of many cytokine receptors was found to be regulated. Self-reactive LPL and IEL from inflamed intestine expressed lower levels of IL-7 receptor a (IL7ra) than the control lymphocytes (Fig. 8 B). A reduced expression of IL-7ra is discussed in the context of regulatory T cells (Gavin et al., 2002; Walkers et al., 2003). Another important cytokine receptor found to be differentially expressed is the IL-6 receptor a (IL-6ra). This receptor is highly expressed on lymphocytes in IBD. Anti-IL-6 receptor monoclonal antibody treatment has been shown to inhibit leukocyte recruitment and promote T cell apoptosis in a murine model of Crohn' disease (Ito et al., 2002). However, in our mouse model IL-6ra expression was decreased in IEL of VILLIN-HA x TCR-HA double transgenic mice (Fig. 8 F). A variety of the chemokine receptors and chemokine ligands was regulated in double transgenic mice. RNA levels of the chemokine receptors CCR2 and CCR6 were up-regulated in $6.5^{+}CD4^{+}$ mucosal lymphocytes from double transgenic mice (Figure 8 E and C), both of which have been shown to participate in the development of a mucosal immune response (Cook et al., 2000; Varona et al., 2001). CCR5 expression was down-regulated in LPL and CCR7 in IEL from inflamed tissue (Fig. 8 D and F). Whereas the expression of CCR5 plays an important role in lymphocyte localization within the gut (Agace et al., 2000), CCR7 expression could be detected on memory T cells, whereas activated T cells down-regulate CCR7 expression (Campbell et al., 2001). The chemokine ligands CCL2, CCL6 and CCL19 which are also thought to play a role in the induction of immune responses and IBD (Banks et al., 2003; Otten et al., 2003) were up-regulated upon inflammation in the double transgenic mice (Fig. 8 E and C). In contrast, expression of the chemokine ligands CCL5 and CCL3 were down-regulated (Fig. 8 D and F) despite the fact that these ligands are normally found to be highly expressed in patients with IBD (Scheerens et al., 2001; Banks et al., 2003).

Under healthy conditions, LPL show an increased susceptibility to apoptosis related to the high expression of FAS antigen and also FAS ligand (De Maria et al., 1996). Besides the fact that the percentage of FAS positive LPL is higher when compared to

their peripheral T cell counterparts, also upon FAS ligation, cell death is induced more effectively in LPL, suggesting that they are "death prone" (De Maria et al., 1996). The significance of LPL preprogrammed cell death for intestinal homeostasis is seen in conditions in which this homeostasis is disturbed. T cells isolated from areas of inflammation in Crohn's disease, ulcerative colitis and other inflammatory states manifest decreased CD2 pathway-induced apoptosis (Boirivant et al., 1999). Differences between normal LPL and those generated from inflamed mucosa show that apoptosis-associated genes such as *bax* and *bcl-2* are differentially expressed in normal versus inflamed mucosa. Specifically, increased expression of the anti-apoptitic BCL-2 protein was shown (Ina et al. 1999, Itho et al. 2001). The expression level of apoptosis inducers and inhibitors is also significantly regulated in mucosal T cells from VILLIN-HA x TCR-HA transgenic mice. In LPL, the RNA transcription level of FAS (Tnfsrf6) was down-regulated and by decrease of EGR2 in IEL the expression of FAS ligand in activated T cells was regulated (Fig. 8 D and F). In addition the expression of the mRNA encoding the anti-apoptitic BCL-2 protein by IEL from double transgenic mice was significantly up-regulated (Fig. 8 E). Besides genes involved in apoptosis, prostaglandins play an important role in the maintenance of the intestinal homeostasis. Kabashima et al. (2002) could demonstrate in EP4 knock-out mice that PTGER 4 suppresses colitis, mucosal damage and $CD4^+$ T cell activation in the gut. In LPL of VILLIN-HA x TCR-HA mice the expression level of PTGER4 was decreased which indicates an inflammation in the intestine (Fig 8 D). In contrast PTGS1 (COX-1), which has been shown to reduce arachidonic acid-induced inflammation and indomethacin-indiced gastric ulceration (Langerbach et al., 1995), was up-regulated in IEL of diseased mice (Fig. 8 E). Constitutive PTGS1 expression is believed to mediate prostaglandin dependent gastric protection (Jackson et al., 2000). Tab. 1 summarizes selected genes indicating inflammation or regulation in the intestinal mucosa.

Table 1: Selected genes differentially expressed in LPL and IEL from VILLIN-HA x TCR-HA and TCR-HA mice

	Name	Regulation	Reference	Cluster
LPL and IEL pro-inflammatory	$\alpha_E\beta_7$	↑	Elewaut et al.,1998	A
	S100a6	↑	Timmons et al., 1993	A
	Snx9	↑	Chen et al., 2001	A
	Tnfrsf7	↑	Raedler et al., 1985	A
	CD83	↑	Croft, 2003 te Velde et al., 2003	A
	Tnfrsf9	↑	Croft, 2003	A
LPL pro-inflammatory	Itgb7	↑	Sun et al., 2001	C
	Itga4	↑	Hornquist et al., 1997 Ghosh et al., 2003	C
	IL-5	↑	Podolsky et al., 2002 Fuss et al., 1996	C
	CCL19	↑	Otten et al., 2003	C
	PTGER4	↓	Kabashima et al., 2002	D
	CCR6	↑	Cook et al., 2001	C
IEL pro-inflammatory	CD7	↑	Varona et al., 2001 Allison et al., 1990	E
	BCL2	↑	Trejdosiewicz et al., 1989 Ina et al., 1999	E
	STAT3	↑	Itoh et al., 2001 Lovato et al., 2003	E
	EGR2	↓	Lechner et al., 2002	F
LPL and IEL anti-inflammatory	CST7	↑	Halfon et al., 1998	A
	Areg	↑	Troyer et al., 2001	A
	IL-10	↑	Van Montfrans et al., 2002	A
	IFN-γ	↓	Fiocchi, 1998	B
	IL-7r	↓	Puel et al., 1998	B
LPL anti-inflammatory	Tnfrsf18/GITR	↑	Shimizu et al., 2002	C
	LTβ	↓	Ronchetti et al., 2002 Agyekum et al., 2003	D
	Tnfrsf6/FAS	↓	Bregenholt et al., 2001	D
	CCR5	↓	Boirivant et al., 1999 Agace et al., 2000	D
	IL-17	↓	Nielsen et al., 2003	D
	ICOS	↓	Fujino et al., 2003 Kanai et al., 2000	D
IEL anti-inflammatory	PTGS1/COX1	↑	Langerbach et al., 1995	E
	ANXA1	↑	Jackson et al., 2000 Gold et al., 1996	E
	CCL3	↓	Vergnolle et al., 1995 Banks et al., 2003	F
	CCR7	↓	Campbell et al., 2001	F
	IL-6ra	↓	Ito et al., 2002	F
	ICAM1	↓	Ito et al., 2002	F
	CXCR3	↓	Yuan et al., 2001	F

continued

	Name	Regulation	Reference	Cluster
LPL/IEL pro-/anti-inflammatory	PD1	↑	Dong et al., 2003 Salama et al., 2003	A
	KLRG1	↑	Voehringer et al., 2002 Robbins et al., 2003	C
	CCL5	↓	Scheerens et al., 2002	D
	Lag3	↑	Workmann et al., 2003	E
	Tnfrsf4/OX40	↑	Murata et al., 2002	A

In conclusion, mucosal inflammation in VILLIN-HA x TCR-HA transgenic mice was accompanied by brought changes in the gene expression pattern of auto-reactive LPL and IEL. The profiling revealed differential expression of pro-inflammatory genes, as well as a remarkable set of genes discussed in the context of immune regulation. Because of the huge amount of data generated by global gene expression profiling, only those genes which are known to be involved in mucosal inflammation or immune regulation are discussed here. The entire data set of this microarray experiments is accessible as MIAME format online under www.gbf.de/array/download.

3.6 Adoptive transfer of 6.5^+CD4^+ and 6.5^+CD4^+depleted of $CD25^+$ T cells into VILLIN-HA transgenic mice

Enterocyte specific IEL from VILLIN-HA x TCR-HA transgenic mice show a reduced reactivity to their corresponding antigen and both, LPL and IEL, secrete lower amounts of pro-inflammatory cytokines such as IFN-γ and IL-2 upon antigenic stimulation *in vitro*. The mild pathology of mucosal inflammation suggested the induction of peripheral tolerance mechanisms, which was further underlined by the gene expression pattern of auto-reactive LPL and IEL isolated from the inflamed tissue. The profiling data revealed differential expression of pro-inflammatory genes, as well as genes discussed in the context of immune regulation and regulatory T cells. Thymic derived $CD4^+CD25^+$ T cells constitute a major population of regulatory T cells that are able to inhibit T cell responses both *in vitro* (Thornton & Sevach, 1998; Read et al., 1998) and *in vivo* (Suri-Payer et al., 1998; Read et al., 2000). The ability of regulatory T cells to control autoimmune diseases has sparked much interest in the question how these cells function to control their naïve counterpart. As the data presented here propose the induction of regulatory mechanisms preventing an uncontrolled progression of mucosal

inflammation in the double transgenic VILLIN-HA x TCR-HA mouse model, it was interesting to analyze whether the small proportion of naturally occurring HA-specific $6.5^+CD4^+CD25^+$ regulatory T cells is able to suppress the proliferative and inflammatory capacity of $6.5^+CD4^+CD25^-$ T cells *in vivo*. Therefore, adoptive transfer experiments of 6.5^+CD4^+ and 6.5^+CD4^+ T cells depleted from $CD25^+$ cells were performed in VILLIN-HA mice. Naïve $CD4^+$ and $CD4^+CD25^-$ T cells were isolated from the spleen of TCR-HA mice by negative selection using the AutoMACS. The percentage of 6.5^+CD4^+ T cells was measured by FACS analysis and CFSE labeling was performed. 2 x 10^6 6.5^+CD4^+ or $6.5^+CD4^+CD25^-$ transgenic T cells were injected i.p. into VILLIN-HA transgenic mice. 7 days after adoptive transfer the *in vivo* proliferation of transgenic T cells was investigated as judged by the loss of CFSE dye in HA-specific $CD4^+$ T cells from spleen, MLN, lamina propria and intestinal epithelium (Fig. 9).

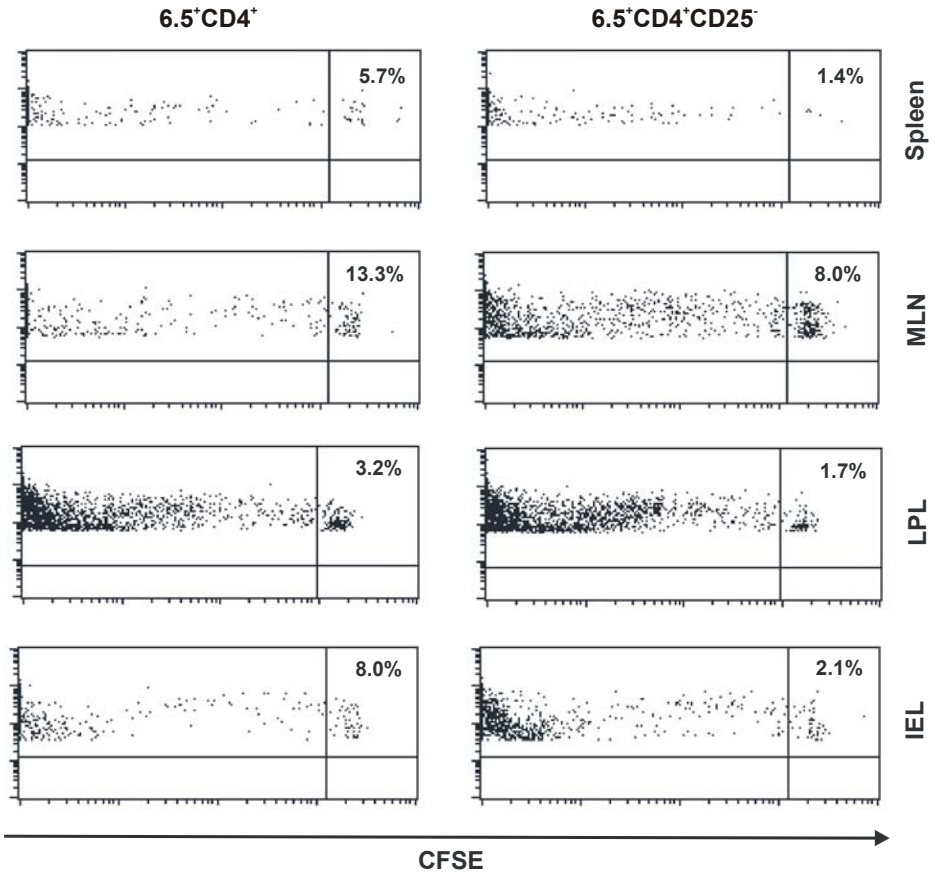


Figure 9: Proliferative response of 6.5^+CD4^+ T cells to tissue derived antigen after adoptive transfer into VILLIN-HA recipients. 2 x 10^6 CFSE labeled $6.5^+CD4^+CD25^-$ or 6.5^+CD4^+ T cells were adoptively transferred into VILLIN-HA recipients. 7 days later cells from spleen, MLN, LP and IE were isolated and stained for 6.5 and CD4 expression. CFSE profiles of gated 6.5^+CD4^+ were estimated.

In all compartments investigated a huge proportion of the $6.5^+CD4^+25^-$ T cells had undergone proliferation in response to gut-derived antigen. The proliferation of 6.5^+CD4^+ T cells was less prominent suggesting that naturally occurring $CD25^+$ T cells suppress antigen specific proliferation of naïve self-reactive $CD4^+$ T cells. To further proof this hypothesis the percentage of 6.5^+CD4^+ transgenic T cells from recipient mice was analyzed. As summarized in Fig. 10, the percentage of 6.5^+CD4^+ T cells was increased in spleen, MLN, LP and IE of VILLIN-HA mice that received 6.5^+CD4^+ T cells depleted of the naturally occurring $CD25^+$ T cells. This effect was most impressive in the LPL and IEL compartments, strongly suggesting the active repression of proliferation of auto-reactive T cells in the gut after antigen encounter by HA specific $6.5^+CD4^+CD25^+$ regulatory T cells.

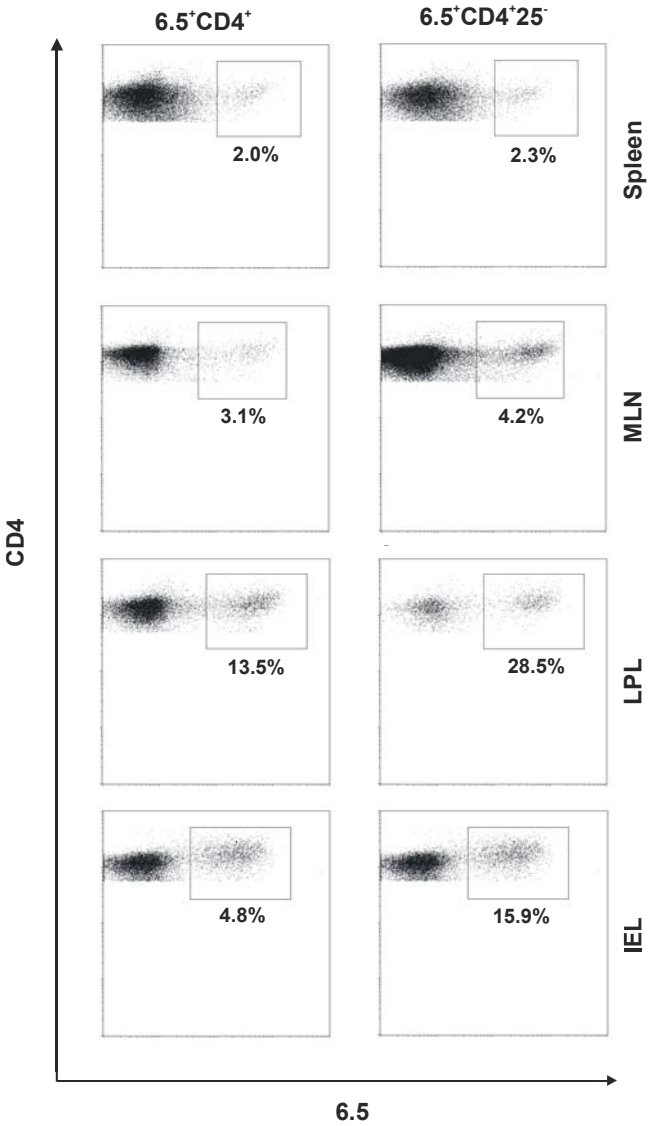


Figure 10: Clonal expansion in response to self-antigen. 2 x 10^6 CFSE labeled 6.5^+CD4^+ or $6.5^+CD4^+CD25^-$ T cells were adoptively transferred i.p. into VILLIN-HA recipients. 7 days later spleen, MLN, LPL and IEL were isolated and stained for 6.5 and CD4 expression to measure the percentage of transgenic T cells in the different compartments.

Taken together, due to inefficient thymic deletion auto-reactive $CD4^+$ T cells migrate to the periphery of VILLIN-HA x TCR-HA double transgenic mice. These cells invade the lamina propria and the intestinal epithelium where they encounter their specific antigen. Antigen contact does not result in uncontrolled inflammation, but in a chronic form of mild enterocolitis, suggesting the induction of peripheral tolerance mechanism counteracting inflammatory processes in the mucosa. These mechanisms obviously include a reduced proliferative capacity of intestinal lymphocytes, changes in the cytokine pattern, as well as brought changes in the gene expression profile of auto-reactive $CD4^+$ T cells from the inflamed gut. In addition, naturally occurring $CD4^+CD25^+$ T cells also seem to be involved in the maintenance of the immunological balance, as they inhibit proliferation of auto-reactive $CD4^+$ T cells in an antigen specific manner.

4 Discussion

The gastrointestinal tract is home to the largest number of lymphocytes in the body as well as being the site where these cells encounter abundant exogenous stimuli. Regulation of the immune response in the intestine is a balance between the need to mount protective immunity towards pathogens and unresponsiveness to harmless antigens present in the intestine, including those derived from resident bacteria. The development of inflammatory bowel disease, encompassing Crohn's disease and ulcerative colitis, provides a dramatic illustration of the consequence of a breakdown in intestinal immune regulation. Basic and clinical studies demonstrated, that IBD share some characteristics with autoimmune diseases, since autoantibodies have been detected in human patients and autoimmune mechanisms clearly contribute to the disease in mice (Mizoguchi et al., 1996; Shanahan et al., 1992). Inappropriate T cell responses towards harmless antigens are responsible for the chronic inflammatory processes leading to intestinal inflammation (Podolsky, 2002; Strober et al., 2002) due to the observation that T cell accumulation in the inflamed tissue occurs.

Mucosal inflammation and tissue damage is predominately mediated by cellular lysis and secretion of perforin by $CD8^+$ T cells (Kagi et al., 1995; Kagi et al., 1996). Additionally, the secretion of inflammatory cytokines like TNF-α potentiates the inflammatory process by enhancing infiltration of mononuclear cells. More recently it was shown that $CD4^+CD45RB^{high}$ T cells are capable to initiate an intestinal inflammation in lymphopenic mice (Dohi et al., 2003). Responsible for regulation and control of the intestinal inflammatory processes are different types of regulatory T cells, Th3 cells, $CD4^+CD25^+$ or $CD4^+CD45RB^{low}$ T cells or $CD8^+$ suppressor T cells (Miller et al., 1992; Sakaguchi et al., 2000; Roncarolo & Levings, 2000; Mowat, 1987). These regulatory cells induce immunosupression in surrounding T cells most likely by secretion of regulatory cytokines such as IL-10 or TGF-β and inhibit inappropriate immune responses towards harmless mucosal antigens (Ludviksson et al., 2000; Strober et al., 1998).

Despite the fact that T cells with an autoaggressive character are involved in the development of intestinal inflammation, it has been difficult to identify self-proteins that may play a role in the etiology or chronicity of IBD and to asses the impact of antigen-specificity. An important aim of this study was to test the hypothesis that antigen-specific $CD4^+$ T cell recognition of a single epithelial self antigen is sufficient to trigger

an inflammatory cascade resulting in histological manifestation in the intestine and investigate whether regulatory mechanisms may suppress inflammation and maintain homeostasis. To analyze the immunological and molecular mechanisms of antigen specific $CD4^+$ T cell response in chronic mucosal inflammation, a transgenic mouse expressing hemagglutinin (HA) in enterocytes of the intestinal epithelium was generated (Templin et al., submitted). Concomitant expression of HA and a MHC class II-restricted T cell receptor specific for HA in VILLIN-HA x TCR-HA mice resulted in an autoimmune mediated chronic intestinal inflammation.

Autoimmune diseases are believed to be under complex genetic regulation, but all require some form of escape from self-tolerance. In VILLIN-HA x TCR-HA double transgenic mice 6.5^+CD4^+ transgenic T cells could be detected in the peripheral lymphatic organs including spleen and MLN (Fig. 1). This finding was not unexpected, as it has been described previously that expression of the HA-antigen in pancreas (Degermann et al., 1994; Sarukhan et al., 1998) and in hematopoetic cells (Lanoue et al., 1997) of INS-HA x TCR-HA and IgHA x TCR-HA double transgenic mice does not lead to complete deletion of 6.5^+ T cells. A possible explanation for the escape from central tolerance might involve coexpression of two different T cell receptors by the same cell. Due to allelic inclusion of TCRα genes self-reactive T cells may leave the thymus resulting in induction of autoimmunity in the periphery (Sarukhan et al., 1998). To exclude that self-reactive peripheral T cells were rendered in an anergic state, we compared the proliferative capacity of transgenic T cells from single and double transgenic mice. Enterocyte-specific expression of HA in VILLIN-HA x TCR-HA did not lead to tolerance induction in the periphery as no differences in their capacity to proliferate upon stimulation with their cognate peptide could be observed (Fig. 2). Flow cytometry analysis revealed that 6.5^+CD4^+ T cells from VILLIN-HA x TCR-HA exhibited an activated phenotype since frequencies of 6.5^+CD4^+ T cells expressing the activation markers CD69 and CD25 in spleen and MLN of these mice were significantly increased as well as the expression of CD45RB and CD62L was reduced (Fig. 3), indicating that HA-specific $CD4^+$ T cells encountered their specific antigen which is expressed exclusively by intestinal epithelial cells.

Immunohistochemistry of the intestine of VILLIN-HA x TCR-HA double transgenic mice was performed to estimate the grade of intestinal inflammation. As demonstrated

in Fig. 4, double transgenic mice showed a mild autoimmune enterocolitis. This was accompanied by infiltration of $CD3^+$ lymphocytes in the LPL and IEL compartment and development of lymph edema. However, tissue damage to the epithelial layer could not be observed, indicating that the immunological balance in double transgenic mice stands on the edge, where on the one hand activation and infiltration of lymphocytes in the intestine occurs, but on the other hand regulatory mechanisms seem to counteract uncontrolled progression of intestinal inflammation.

To characterize the inflammatory processes in the intestine of VILLIN-HA x TCR-HA mice in more detail 6.5^+CD4^+ LPL and IEL were analyzed according to their proliferative capacity upon antigenic stimulation and secretion of pro- or anti-inflammatory cytokines. When mucosal lymphocytes are stimulated via the T cell receptor they normally respond only poorly and activation seems to be dependent on CD2/CD28 stimulation to result in proliferation and cytokine secretion (Boirivant et al., 1999; Targan et al., 1995). Interestingly, IEL and LPL from TCR-HA mice as well as LPL from VILLIN-HA x TCR-HA transgenic mice proliferate in an antigen dose dependent manner. In contrast, the specific proliferative capacity of IEL from double transgenic mice was abrogated with a high background proliferation even without antigenic stimulation, thus resembling the normal phenotype of mucosal lymphocytes (Fig. 5). The cytokine profile in inflammatory bowel disease shows some characteristic differences depending on the kind of disease. Crohn's disease is associated with a T_H1 cytokine pattern, characterized by INF-γ, TNF-α, and IL-12 secretion (Fiocchi, 1998; Elson, 2000). In ulcerative colitis, the cytokine profile is less restricted, it is not resembling a T_H1 response and appears to be a modified T_H2 response associated with cytokines such as IL-5 and IL-10 (Fiocchi, 1998; Sands, 2000). In the VILLIN-HA x TCR-HA transgenic mouse model antigen stimulated 6.5^+CD4^+ LPL and IEL from diseased mice secreted lower amounts of INF-γ and IL-2 upon *in vitro* stimulation compared to control mice (Fig. 6 and 7). These data suggested a suppression of pro-inflammatory mediators in the intestine of double transgenic mice. However, the basal level secretion of TNF-α, MCP-1, and IL-6, which are all important mediators in the induction of IBD, was considerably increased in LPL and IEL from double transgenic mice (Fig. 6 and 7). This disagreement in the cytokine secretion of transgenic LPL and IEL from VILLIN-HA x TCR-HA mice may denote a steady state between regulatory and pathological mechanisms being active in the intestine. To consider this hypothesis

in more detail, global gene expression analysis of HA-specific LPL and IEL which were isolated directly from the intestine of VILLIN-HA x TCR-HA mice or from TCR-HA control mice was performed.

A heterogeneous set of genes differentially expressed in auto-reactive $CD4^+$ T cells was identified. As expected, many of the genes found to be regulated have been described earlier in the context of intestinal inflammation. Some pro-inflammatory genes were specifically regulated in both LPL and IEL. In agreement with published data, the integrin $\alpha_E\beta_7$ expression was up-regulated in 6.5^+CD4^+ LPL and IEL from inflamed tissue of VILLIN-HA x TCR-HA transgenic mice when compared with cells form control mice. It has been shown that changes of $\alpha_E\beta_7$ expression in Crohn's disease and ulcerative colitis patients versus controls are of pathological relevance and that this may be one of the earliest events in the pathogenesis of this disease (Elewaut et al., 1998). A wide variety of members of TNF receptor superfamily were differentially expressed on LPL and IEL from double transgenic mice. Especially, Tnfrsf7 and Tnfrsf9 were up-regulated on LPL and IEL from inflamed tissue. This molecules are known to be expressed in elevated numbers of peripheral lymphocytes in inflammatory bowel disease (Raedler et al., 1985; Croft, 2003). LPL and IEL do not resemble a homogenous T cell population, each population has an own phenotype with specialized function. According to this characteristics, the gene expression profile of these cells may differ. Indeed, many genes were found to be exclusively regulated in LPL of VILLIN-HA x TCR-HA transgenic mice. Although $\alpha_E\beta_7$ was up-regulated in both LPL and IEL, expression of various other integrins was significantly increased only in LPL from inflamed tissue. Integrins are involved in lymphocyte homing to the intestinal mucosa and it has been demonstrated that their expression is often enhanced upon intestinal inflammation (Sun et al., 2001; Hornquisst et al., 1997; Podolsky et al., 1993). Also IL-5 secretion was significantly up-regulated in LPL from VILLIN-HA x TCR-HA mice. This cytokine plays a key role in the induction of ulcerative colitis (Fuss et al., 1996). Besides genes exclusively regulated in LPL, many pro-inflammatory genes exist, which expression level is only changed in the IEL of double transgenic mice. One example is the expression of the CD7 surface molecule. In line with published data which show that the frequency of $CD7^+$ T cells is significantly increased in inflammatory bowel disease, the expression of CD7 in IEL of VILLIN-HA x TCR-HA mice was up-regulated. In addition, the expression level of STAT 3 was increased in IEL. STAT3 has been shown

to be directly linked to secretion of IL-6 in inflammatory bowel disease (Wang et al., 2003). This is in accordance with the finding that LPL and IEL from VILLIN-HA x TCR-HA secrete higher amounts of IL-6 compared to control cells (Fig. 8). Intestinal inflammation is often initiated by a failure of mucosal lymphocytes to undergo preprogrammed cell death (De Maria et al., 1996) In agreement with this, the anti-apoptotic *bcl*-gene expression was significantly up-regulated in IEL of the inflamed tissue. In addition, a decreased EGR2 expression level was found in IEL from double transgenic mice, a factor known to expression of FAS ligand in activated T cells (Lechner et al., 2001). Many other genes were found to be significantly up- or down-regulated in the inflamed intestine when compared with healthy donors, such as CD83, CCL19, PTGER4 or CCR7 and numerous others which have been discussed in the context of inflammatory bowel disease (te Velde et al., 2003; Otten et al., 2003; Kabashima et al., 2002; Campbell et al., 2001).

In addition to genes that are associated with intestinal inflammation, also a large number of genes previously been described to play a role in immune regulation in the intestine have been identified. The major finding was, that the expression of IL-10 and IFN-γ, both of which are mediators playing important roles in the regulation of progression of IBD, were significantly regulated in IEL and LPL of diseased mice. IL-10 was highly up-regulated in LPL and IEL from double transgenic mice compared to control mice. Using a murine knock-out model it has been shown, that IL-10 prevents the development of intestinal inflammation (Kuhn et al., 1993). Furthermore, the application of IL-10 to diseased mice abrogated clinical signs or suppressed the inflammation in the intestine (Steidler et al., 2000). In addition, INF-γ, which plays a key role in the induction of IBD, was down-regulated in mucosal lymphocytes of VILLIN-HA x TCR-HA transgenic mice. Furthermore, the expression of other pro-inflammatory cytokine like LT-β and IL-17 in LPL from double transgenic mice was also down-regulated. Recently, it has been shown that Tnfrsf18 (GITR) is predominantly expressed on $CD4^+CD25^+$ regulatory T cells (Shimizu et al., 2002). This member of the TCR receptor superfamily was significantly up-regulated in LPL of double transgenic mice. Also IEL showed differential gene expression resembling the induction of regulatory mechanisms to maintain homeostasis. Constitutive PTGS1 expression is believed to mediate prostaglandin dependent gastric protection (Jackson et al., 2000). In IEL of VILLIN-HA x TCR-HA transgenic mice, prostaglandin expression was up-regulated. In contrast, genes involved in IBD induction like CCR7, IL-6a or

ICAM were down-regulated in IEL. In summary, mucosal inflammation in the intestine of VILLIN-HA x TCR-HA transgenic mice was accompanied by brought changes in the gene expression pattern of LPL and IEL. Profiling revealed differential expression of pro-inflammatory genes, as well as a remarkable set of genes discussed in the context of immune regulation.

The mild form of mucosal inflammation suggested the induction of peripheral tolerance mechanisms, a hypothesis which was further underlined by the gene expression pattern of LPL and IEL from VILLIN-HA x TCR-HA transgenic mice. The induced expression of a wide variety of genes involved in regulatory processes suggested the induction of regulatory T cells in the intestine counteracting the uncontrolled progression of autoimmune disease. In addition to T_{reg} cells induced in an antigen-specific manner in the periphery of double transgenic mice, every individual harbors a small population of naturally occurring, thymus derived $CD4^+CD25^+$ T cells that are able to inhibit T cell responses both *in vitro* (Takahashi et al., 1998; Read et al., 2000) and *in vivo* (Suri-Payer et al., 1998; Read et al., 2000). Adoptive transfer experiments into VILLIN-HA transgenic mice demonstrated that this small proportion of naturally occurring regulatory T cells was sufficient to reduce the profilerative capacity of naïve transgenic T cells *in vivo* (Fig. 9). In most of the published transfer experiments done to characterize the properties of regulatory T cells, the hosts are lymphopenic and the transferred T cell subsets are polyclonal with unknown antigen specificity (Asseman et al., 2003; Maloy et al., 2003;). Therefore, physiological regulatory functions cannot be distinguished easily from effects that are caused by homeostatic proliferation and clonal expansion of transferred cells (Bach, 2003; Barthlott et al., 2003). Transfer experiments into VILLIN-HA mice are based on the use of animals with an intact immune cell repertoire. Thus, the results summarized in Fig. 10 demonstrated that the effect of transferred T cells on antigen specific proliferation and clonal expansion is due to suppressor function of T_{reg} cells and not a result of homeostatic proliferation in a lymphopenic host. Furthermore, as the transferred T cells represent a monoclonal subset with known antigen specificity to an autoantigen, the obtained results resemble an interaction between antigen induced regulatory T cells and naturally occurring T_{reg} cells in the VILLIN-HA x TCR-HA double transgenic mouse model.

Further studies are needed to clarify the role of regulatory T cells in the development of chronic intestinal inflammation in VILLIN-HA x TCR-HA transgenic mice. The mild

chronic intestinal inflammation represents an unbalance between inflammatory processes and mucosal immune regulation. These data provide novel insights into pathogenic mucosal T cell responses in chronic intestinal inflammation and will permit to carefully dissect the mechanisms by which enterocyte specific LPL and IEL regulate or prime intestinal inflammation.

5 Summary

Chronic inflammatory bowel disease could be the consequence of an antigen specific dysregulated T cell response with the expansion of T cells and the induction of immunopathology. The immunological and molecular mechanisms of antigen specific $CD4^+$ T cell response in chronic mucosal inflammation was analyzed using a transgenic mouse expressing hemagglutinin (HA) in enterocytes of the intestinal epithelium. Concomitant expression of HA and a MHC class II-restricted T cell receptor specific for HA resulted in an autoimmune mediated chronic inflammation. This inflammation was accompanied by activation of peripheral HA specific lymphocytes and lymphocytic infiltration in the lamina propria and intestinal epithelium. The mild form of mucosal inflammation suggested the induction of peripheral tolerance mechanisms. These mechanisms were studied in more detail. Extensive immunological characterization of self reactive LPL and IEL isolated from the inflamed intestine was performed by cellular assays and global gene expression profiling. Enterocyte specific LPL show a dose dependent proliferative response upon antigenic stimulation, whereas the proliferative capacity of IEL was reduced. After *in vitro* stimulation, mucosal lymphocytes from diseased secreted lower amounts of the pro-inflammatory cytokines INF-γ and IL-2, but the secretion of TNF-α, MCP-1 and IL-6 was increased. Moreover, mucosal inflammation was accompanied by brought changes in the gene expression pattern of LPL and IEL. The profiling revealed differential expression of pro-inflammatory genes, as well as a remarkable set of genes discussed in the context of immune regulation. Transfer of naïve 6.5^+CD4^+ and $6.5^+CD4^+CD25^-$ transgenic T cells into VILLIN-HA transgenic mice demonstrated the regulatory potential of the small proportion of naturally occurring $CD4^+CD25^+$ T cells, that might act in concert with peripherally induced regulatory T cells to prevent uncontrolled progression of the disease. The VILLIN-HA x TCR-HA double transgenic mouse model will permit to carefully dissect the mechanisms by which enterocyte specific LPL and IEL regulate or prime a chronic intestinal inflammation and to identify target molecules useful for therapeutic approaches in the field of intestinal diseases.

CHAPTER III

Modulation of mucosal immune responses using bacterial cell surface display of a functional T cell epitope

Modulation of mucosal immune response using bacterial cell surface display of a functional T cell epitope

1 Background

IBD models support a central role for dysregulated $CD4^+$ T cell response to enteric bacterial flora as a common disease mechanism. The mechanisms by which the bacterial flora stimulates colitis in susceptible hosts is unknown and is likely to be multifactorial. A recent study showed that colitis can be caused by reconstituting SCID mice with $CD4^+$ T cell lines established from C3H/HeBir mice exposing the mice to their fecal extracts (Brimnes J. et al., 2001). Additionally, reconstitution of SCID mice with naïve $CD4^+$ T cells specific to OVA or the transfer of those cells into BALB/c mice followed by colonization of these mice with OVA expressing *E.coli* led to wasting disease (Yoshida et al., 2001; Yoshida et al., 2002). In contrast, Iqbal et al. (2002) published a colitis model based on the use of OVA-producing *E.coli* and the transfer of T_H1 or T_H2 OVA-specific $CD4^+$ T cells into $RAG2^{-/-}$ mice. No colitis was observed in mice that received naïve $CD4^+$ T cells specific for the antigen produced by luminal *E.coli* in the intestine. Thus, different mouse models used to study the role of bacterial antigen on the induction of IBD lead to controversial results. Growing evidence suggests that inflammatory bowel disease may be associated with a dysregulated mucosal immune response towards indigenous microbial antigens in a genetically susceptible host with the breakdown of epithelial integrity or loss of tolerance (Elson et al., 1995; Strober et al., 1998). However, it has been shown that not all members of the microflora are necessarily pathogenic in IBD. Evidence has emerged that probiotics ameliorate, rather than induce inflammation (Madsen et al., 1999). Possible mechanisms of probiotic action in inflammatory bowel disease include the production of antimicrobial factors, competitive interaction with pathogens, and crosstalk with the host epithelium (Shanahan, 2000). Thus, on the one hand the intestinal microflora including probiotics have beneficial effects on the host immunity and on the other hand bacteria present in the gut lumen are able to induce gut inflammation. These findings highlight the existence of a sensitive balance between anti- and pro-inflammatory factors in the gut.

2 Aims of the study

To investigate the possibility of influencing the immunological balance in the intestine by manipulation of the microflora with bacteria expressing a specific antigen, the probiotic *E.coli* NISSLE 1917 strain was transformed to express the HA110-120 peptide at the bacterial surface. To answer the question, whether $CD4^+$ T cells specific for the bacterially expressed HA in the mouse gut have the potential to initiate immunopathogenic mechanisms leading to mucosal inflammation, extensive analyses on the interaction of bacterial associated antigen in the intestine and HA-specific $CD4^+$ T cells should be performed:

- Characterization of the antigenic potential of *E.coli* NISSLE-HA110-120 *in vitro* as well as *in vivo.*

- Determination of the impact of bacterial antigen expression in the intestine on migration, activation and clonal expansion of antigen specific $CD4^+$ T cells.

- Studies should be performed in TCR-HA, BALB/c, as well as in lymphopenic $RAG1^{-/-}$ mice adoptively transferred with antigen-specific $CD4^+$ T cells.

- To define the role of an intact epithelial barrier on the modulatory effect of *E.coli* NISSLE, experiments should be carried out in healthy mice with a completely intact barrier or in diseased mice with a defect intestinal barrier obtained by the treatment with chemically agents.

- Finally, the potential of *E.coli* NISSLE as carrier organism for gut specific delivery of biological important molecules in the context of intestinal inflammation should be studied.

3 Results

3.1 Generation and characterization of *E.coli* NISSLE 1917 expressing the HA110-120 peptide at their surface

To study the influence of luminal bacterial antigen on the development of a mucosal T cell response in the mouse intestine, bacteria were generated which express a specific antigen at their surface. For efficient export and surface expression of passenger proteins through the inner and outer bacterial membrane the adhesin-involved-in-diffuse-adherence (AIDA) autotransporter system was used. The AIDA autotransporter is synthesized as precursor comprising of a N-terminal leader peptide (SP) that targets the protein to the periplasm, the C-terminal AIDAc, which inserts into the outer membrane and transports the N-terminally linked AIDA-I to the bacterial surface (Konieczny et al., 2000). The MHC class II HA110-120 epitope of the influenza virus A/PR/8/34 (HA) was incorporated into permissive sites of the AIDAc translocator module (Fig. 1A). The construction of the HA110-120/AIDA fusion protein should result in the surface display of the HA110-120 peptide (Fig. 1B). The plasmid harboring the gene for the AIDA autotransporter protein also encodes for an ampicillin resistance gene. As bacterial carrier strain *E.coli* NISSLE 1917 was chosen. This apathogenic strain is part of the commensal microflora, it is characterized by its excellent colonization properties in the gut and is used as probiotics in the biological therapy of intestinal disease.

To assess the functionality of the AIDA autotransporter system for the surface expression of the HA110-120 epitope, immunolabeling experiments were done. *E.coli* NISSLE-HA110-120 were surface labeled with mouse antibody raised against the HA110-120 epitope and a goat anti mouse CyTM3 conjugated secondary antibody. Fluorescence microscopy showed that *E.coli* NISSLE-HA110-120 expressed the antigenic epitope at their surface (Fig. 1C I). *E.coli* NISSLE transformed with a plasmid encoding for the AIDA autotransporter served as negative control (Fig. 1C II). An important question to answer was whether the presentation of the HA110-120 T cell epitope at the surface of bacteria would result in the stimulation of corresponding T cells *in vitro*. To this end, peritoneal macrophages were exposed to *E.coli* NISSLE-HA110-120 and than tested *in vitro* for stimulation of TCR-HA transgenic T cells. As shown in Fig. 1D, HA-specific T cells proliferated *in vitro* in response to macrophages pulsed with bacteria expressing the HA110-120 peptide, but not with control bacteria. Taken together, transformed *E.coli* NISSLE produced the HA110-120 peptide and presented

this epitope at their surface. This epitope is potently immunogenic for TCR-HA T cells *in vitro*.

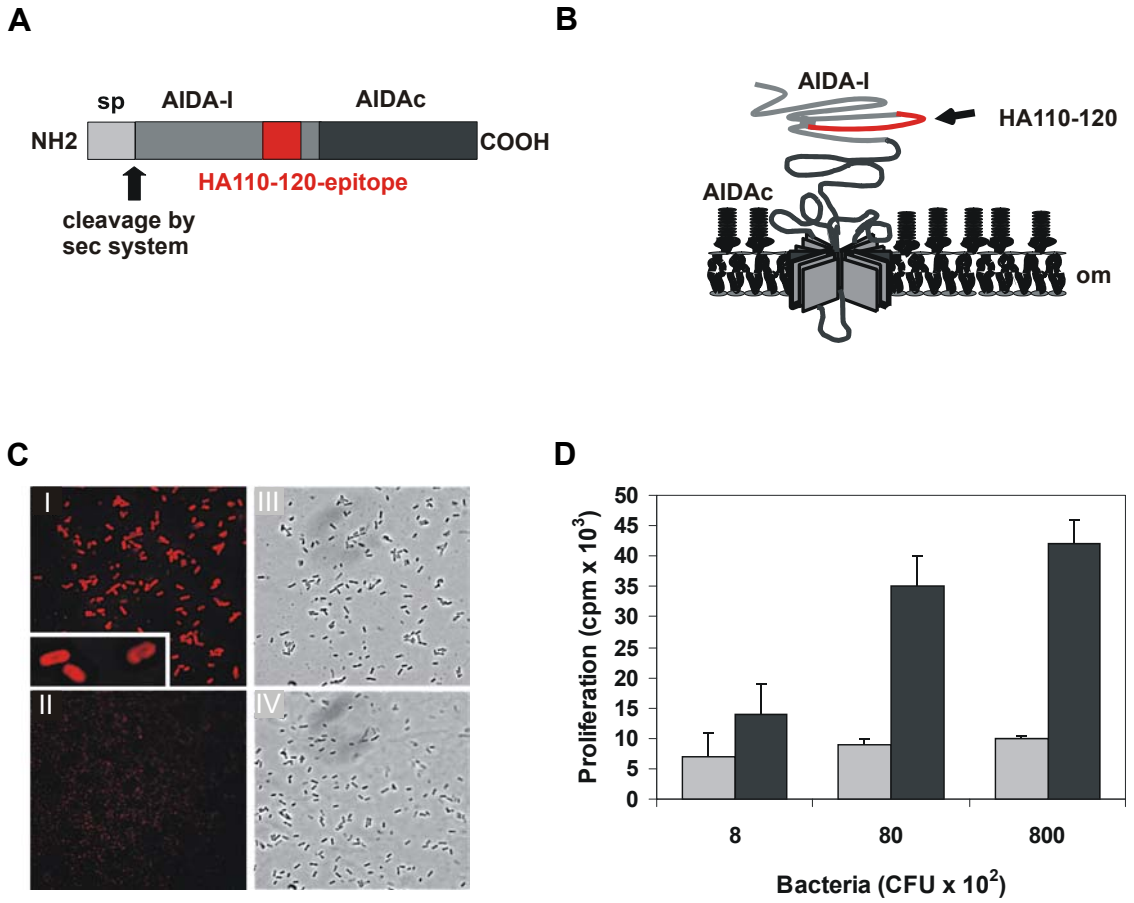


Figure 1: Expression and immunogenicity of the MHC class II peptide HA110-120. (A) Schematic representation of the AIDA (adhesine involved in diffuse adherence) autotransporter and the use of this autotransporter for the presentation of the HA110-120 peptide at the surface of *E.coli* NISSLE. HA110-120 was integrated into the extracellular part of the AIDAc domain. **(B)** Model of the integration of AIDAc in the outer membrane and transport of the N-terminally linked AIDA-I and the integrated HA110-120 to the bacterial surface. **(C)** Immunofluorescence microscopy of *E.coli* NISSLE-HA110-120 (I) and *E.coli* NISSLE control strain (II) using an antibody specific for the HA110-120. III and IV show the phasecontrast of the transformed *E.coli* NISSLE. **(D)** Proliferation of HA110-120 specific T cells. Peritoneal cells were incubated with HA expressing bacteria for 5 h. Thereafter, HA specific T cells were added in medium containing antibiotics. After 48 h T cells were labeled with ^{3}[H]-thymidine and proliferation was measured by thymidine incorporation.

The most important requirement for the use of *E.coli* NISSLE-HA110-120 to study the effect of bacterial antigen expression on the mucosal immune system is that the bacteria have the potential to stimulate a specific T cell response. The capacity of recombinant

E.coli NISSLE-HA110-120 to stimulate TCR-HA transgenic T cells *in vivo* was assessed by adoptive transfer experiments. BALB/c mice that received 2.5 x 10^6 CFSE labeled TCR-HA $CD4^+$ T cells were inoculated i.p. with either PBS, 10^8 control *E.coli* NISSLE or 10^8 *E.coli* NISSLE expressing the HA110-120 peptide. Two days later the *in vivo* proliferation of TCR-HA transgenic T cells was measured in spleen and MLN by loss of CFSE labeling. Injection of HA110-120 expressing bacteria resulted in a complete loss of CFSE in nearly all HA-specific T cells in spleen and MLN, indicating that the HA expressing bacteria are able to stimulate a HA-specific $CD4^+$ T cell response *in vivo* (Fig. 2). Injection of PBS and control bacteria also resulted in T cell proliferative response but to a significant lower extend. These data indicate that the *E.coli* NISSLE-HA110-120 have the potential to stimulate antigen specific responses *in vitro* as well as *in vivo*.

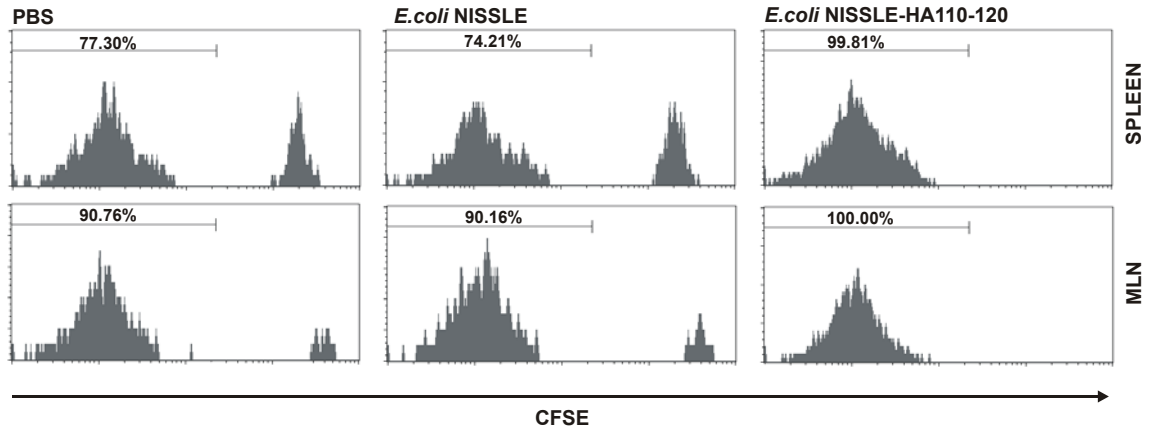


Figure 2: *In vivo* proliferation of HA-specific $CD4^+$ T cells after injection of HA110-120 expressing *E.coli* NISSLE. BALB/c recipients of CFSE labeled HA-specific $CD4^+$ T cells were injected i.p. with PBS (left panels), 10^8 control *E. coli* NISSLE (middle panels) or 10^8 *E. coli* NISSLE-HA110-120 (right panels). Two days later splenocytes and mesenteric lymph node cells were stained with anti-CD4 and anti-6.5. 6.5^+CD4^+ cells were gated and the CFSE labeling of the transgenic cells is shown as histogram.

3.2 Characterization of the primary T cell response induced *in vivo* by *E.coli* NISSLE expressing the HA110-120 peptide in the gut

To directly evaluate the influence of bacterial antigen expression in the gut lumen on the corresponding transgenic T cells *in vivo* TCR-HA mice were colonized with *E.coli* NISSLE-HA110-120 or control bacteria by a single oral application of 2 x 10^{15} CFU per

mouse. To ensure stable colonization of the intestine, 0.3 mg/ml ampicillin were added to the drinking water of the mice and the CFU/g feces were determined during the time of the experiment. Two weeks after oral application of the bacteria the mice were sacrificed and the percentage of HA-specific $6.5^{+}CD4^{+}$ T cells in spleen, MLN, LPL, and IEL was analyzed (Fig. 3).

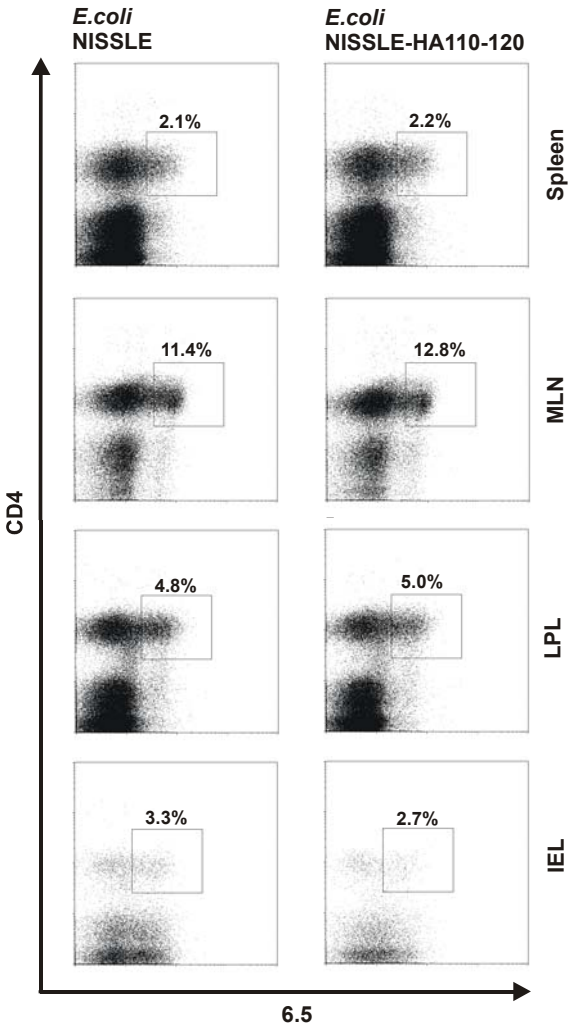


Figure 3: *E.coli* NISSLE expressing the HA110-120 peptide in the gut of TCR-HA mice do not induce expansion of HA-specific $CD4^{+}$ T cells. TCR-HA mice were inoculated either with *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After two weeks the mice were sacrificed and flow cytometry was done to determine the percentage of transgenic T cells in spleen, MLN, LPL and IEL.

In all compartments tested no significant differences in the percentage of transgenic T cells could be observed in mice colonized with *E.coli* NISSLE-HA110-120 compared to the control strain, indicating that luminal antigen expression obviously did not result in clonal expansion of $6.5^{+}CD4^{+}$ T cells and also not in increased migration of HA-specific $CD4^{+}$ T cells to the intestine. TCR stimulation should result in up-regulation of CD25 and CD69 as well as down-regulation of CD45RB and CD62L. To investigate, whether

transgenic T cells are activated by luminal bacteria expressing the HA110-120 peptide, the activation status of HA-specific transgenic T cells was assessed (Fig. 4).

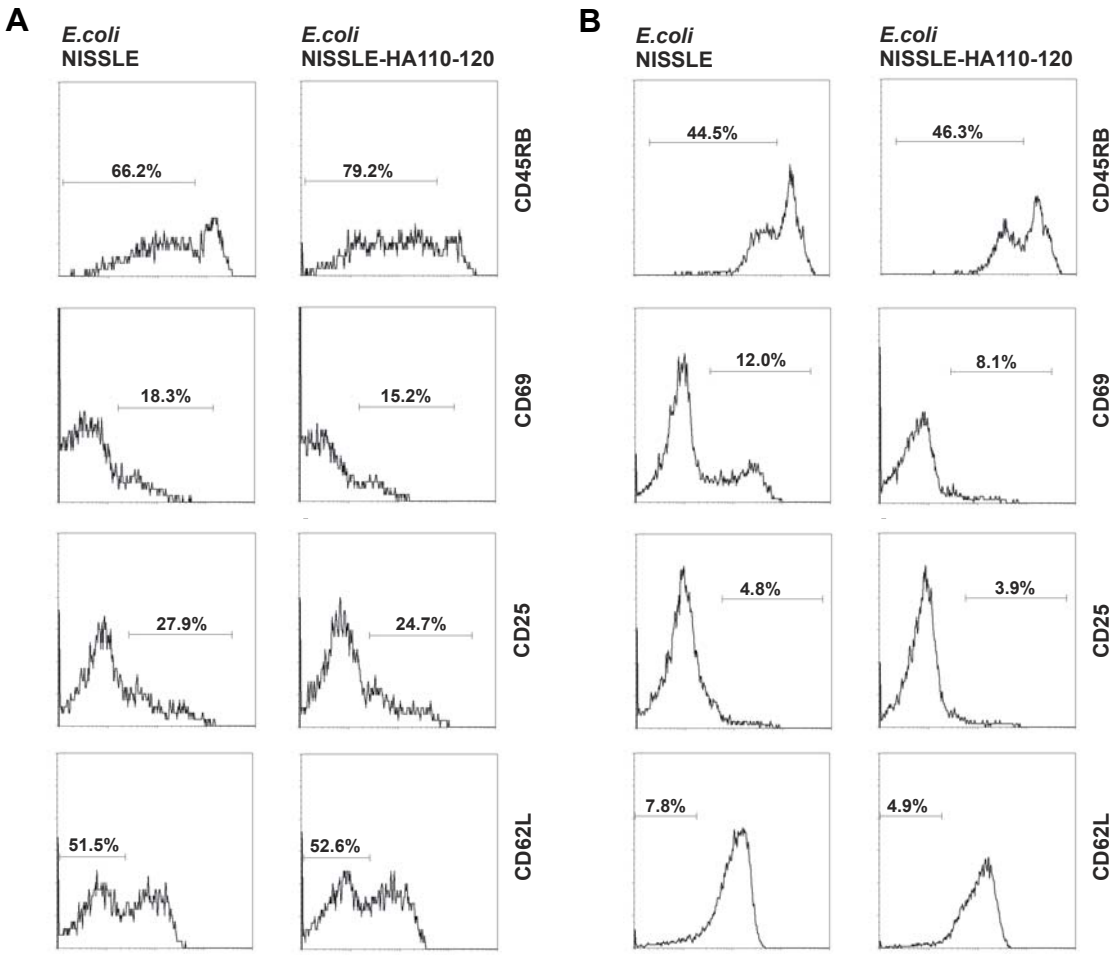


Figure 4: HA-specific $CD4^+$ transgenic T cells from colonized TCR-HA mice showed no changes in the activation pattern. TCR-HA mice were inoculated either with *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After two weeks the mice were sacrificed and flow cytometry on 6.5^+CD4^+ gated cell from spleen **(A)** and MLN **(B)** was done to determine activation profile.

HA-specific T cells in spleen and MLN of colonized TCR-HA mice showed no changes in the expression of CD45RB, CD69, CD25 and CD62L when comparing the phenotype of cells isolated from mice colonized with *E.coli* NISSLE-HA110-120 and control bacteria. To exclude the possibility that the observed unresponsiveness of transgenic T cells is due to inappropriate duration of the colonization periods, a kinetic was done including colonization of TCR-HA mice for one, two, three and four weeks as well as for 6 month. In any case, no significant effect of bacterial antigen expression in the gut

on migration, clonal expansion and the activation status of HA-specific $CD4^+$ T cells could be observed.

3.3 Bacterial colonization of BALB/c mice adoptively transferred with HA-specific $CD4^+$ did not result in clonal expansion of 6.5^+CD4^+ transgenic T cells

Naïve HA-specific $CD4^+$ cells continuously mature in the thymus of TCR-HA mice which then migrate into peripheral organs. This naïve T cells might conceal the activated phenotype of a comparably small number of antigen experienced T cells. To exclude that this fact might be responsible for an undetectable immune response to the bacterially expressed antigen in TCR-HA mice, adoptive transfer of a defined amount of 6.5^+CD4^+ transgenic T cells into BALB/c mice was done. 2 x 10^6 transgenic T cells were transferred i.p. into BALB/c mice followed by the colonization of the recipients with *E.coli* NISSLE-HA110-120 or the control strain. At day 7 after oral application of bacteria the mice were sacrificed and the percentage of transgenic T cells in spleen and MLN was measured, as a proliferative response of 6.5^+CD4^+ transgenic T cells should result in an increase of transgenic T cell numbers. However, no significant differences in the number of transgenic T cells in spleen and MLN could be detected in the *E.coli* NISSLE-HA110-120 colonized mice when compared to control mice (Fig. 5).

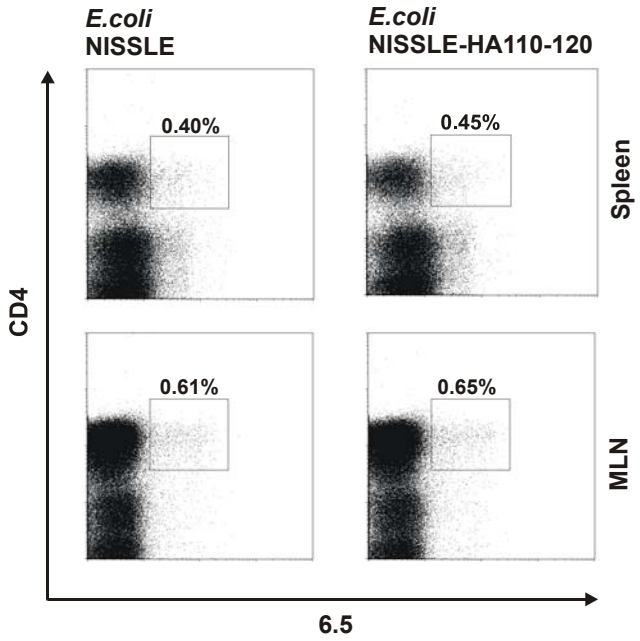


Figure 5: Adoptive transfer of HA-specific $CD4^+$ T cells into BALB/c mice colonized with *E.coli* NISSLE-HA110-120 did not lead to clonal expansion of 6.5^+CD4^+ transgenic T cells. BALB/c mice were injected i.p. with 2 x 10^6 transgenic $CD4^+$ T cells and recipients were inoculated with *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. 7 days later two-color flow cytometric analysis was performed on spleen and MLN cells. The percentage of 6.5^+CD4^+ T cells in the lymphocyte gate was determined.

3.4 Transfer of *in vitro* activated HA-specific CD4$^+$ T cells into BALB/c mice and colonization with *E.coli* NISSLE-HA110-120 did not result in antigen specific immune response

Naïve T cells need a certain antigen threshold for the induction of an immune response against the specific antigen. To exclude the possibility that the antigen concentration produced by the orally applied bacteria in the murine gut was too low to activate T cells, *in vitro* activated HA-specific CD4$^+$ T cells were used for an adoptive transfer to abate the stimulus threshold. Therefore splenocytes of TCR-HA mice were activated with the corresponding HA110-120 peptide for 4 days in culture and the percentage of transgenic T cells as well as their activation status was measured by flow cytometry. 40 % of the splenocytes in culture were 6.5$^+$CD4$^+$ T cells and showed an activated phenotype as indicated by the expression of CD69 and CD25 as well as down-regulation of CD45RB (Fig. 6).

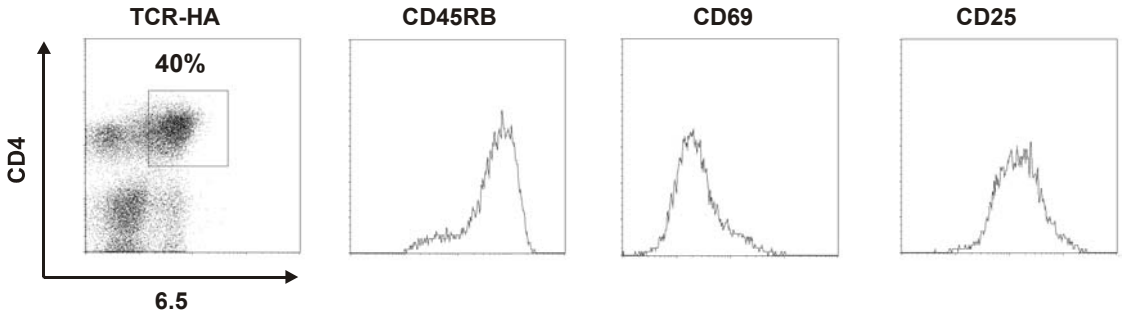


Figure 6: Activated phenotype of 6.5$^+$CD4$^+$ T cells after *in vitro* stimulation. Splenocytes of TCR-HA mice were isolated and activated with 10 µg/ml HA110-120 peptide. After 4 days dead cells were removed by ficoll gradient centrifugation and the viable cells were cultured an additional day. Flow cytometric analysis was performed to determine the percentage of 6.5$^+$CD4$^+$ cells in the lymphocyte population and the activation state of the HA-specific T cells. Histograms were obtained after gating on 6.5$^+$CD4$^+$ T cells.

BALB/c were injected i.p. with 6 x 10^6 activated 6.5$^+$CD4$^+$ transgenic T cells and colonized with *E.coli* NISSLE-HA110-120 or control bacteria. 14 days after adoptive transfer and bacterial colonization the percentage of transgenic T cells was measured in spleen and MLN by flow cytometry. No differences in the percentage could be detected independent of the bacterial strain used for the colonization. 0.39 % TCR-HA transgenic

T cells were found in the spleen and about 0.7 % in the MLN of BALB/c mice previously transferred with activated $6.5^{+}CD4^{+}$ transgenic T cells (Fig. 7A). The activation profile of the transgenic T cells was identical when comparing colonization of transferred mice with *E.coli* NISSLE-HA110-120 and the *E.coli* NISSLE control strain (Fig. 7B). As expected, the activation marker CD69 and CD25 were down-regulated two weeks after transfer. Cells showed a memory T cell phenotype indicated by low expression levels of the CD45RB molecule. Thus, also the adoptive transfer system using activated HA-specific T cells did not support the idea, that luminal antigen produced by bacteria might result in the stimulation of a specific T cell response in mice.

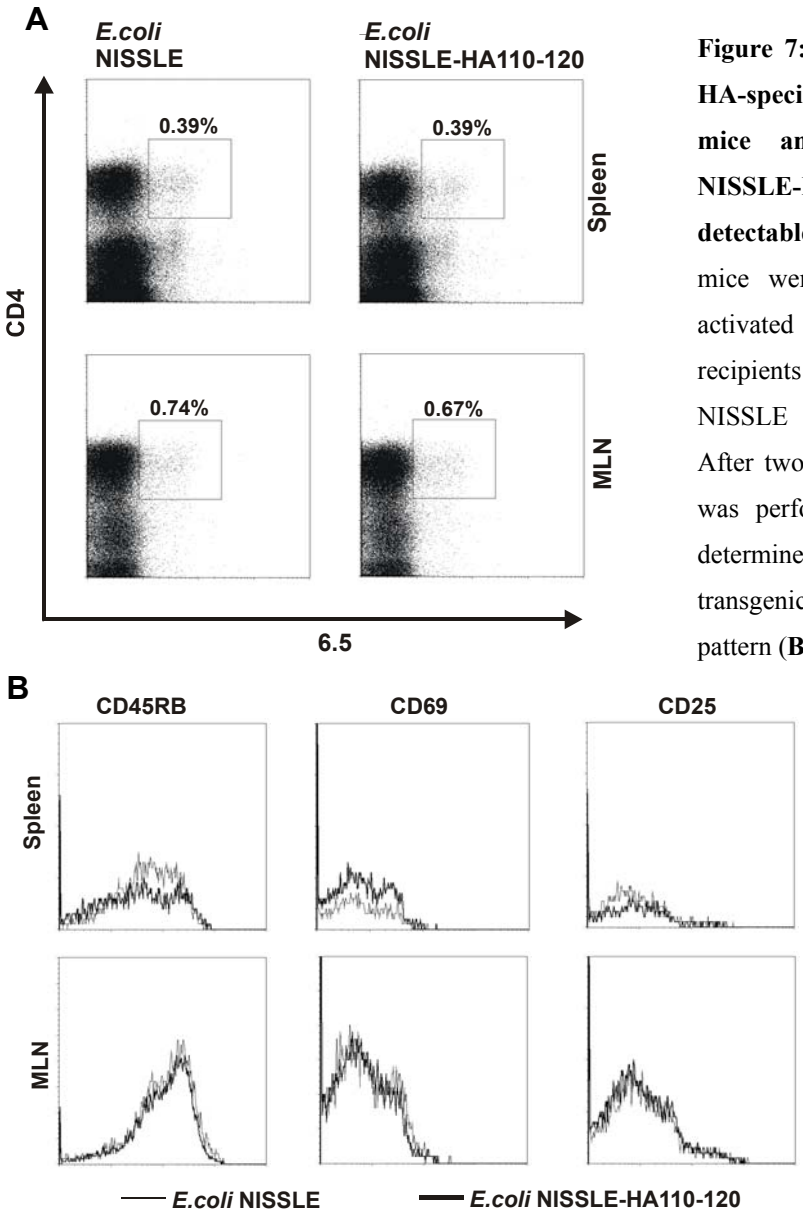


Figure 7: Transfer of *in vitro* activated HA-specific $CD4^{+}$ T cells into BALB/c mice and colonization with *E.coli* NISSLE-HA110-120 does not lead to detectable immune responses. BALB/c mice were injected i.p. with 6 x 10^{6} activated HA specific $CD4^{+}$ T cells and recipients were inoculated with *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After two weeks flow cytometric analysis was performed on spleen and MLN to determine to percentage of $6.5^{+}CD4^{+}$ transgenic T cells (**A**) and their activation pattern (**B**).

3.5 Adoptive transfer of HA-specific $CD4^+$ T cells into *E.coli* NISSLE-HA110-120 colonized RAG-$1^{-/-}$ mice does not induce clonal expansion of transgenic T cells.

It was hypothesized that the balance of the peripheral immune system might be a side effect of normal competition. To clarify whether this natural competition prevents an immune response to the bacterial derived specific antigen in this model, 10^7 transgenic $CD4^+$ T cells were injected i.p. into RAG-$1^{-/-}$ mice and recipients were orally inoculated with *E.coli* NISSLE-HA110-120 or *E.coli* NISSLE control bacteria. RAG$1^{-/-}$ mice are lymphopenic, i.e. these mice lack the T and B cell populations. Three weeks after transfer and bacterial colonization flow cytometric analysis was performed on splenocytes and MLN of the recipient mice. No differences in the percentage of transgenic T cells could be observed in mice colonized with *E.coli* NISSLE-HA110-120 compared to the control animals. In the spleen 11 % and in the MLN 33 % to 37 % of the recovered lymphocytes were 6.5^+CD4^+ transgenic T cells. Thus, the normal competition is not the cause of failure of immune response against antigens of bacterial gut flora.

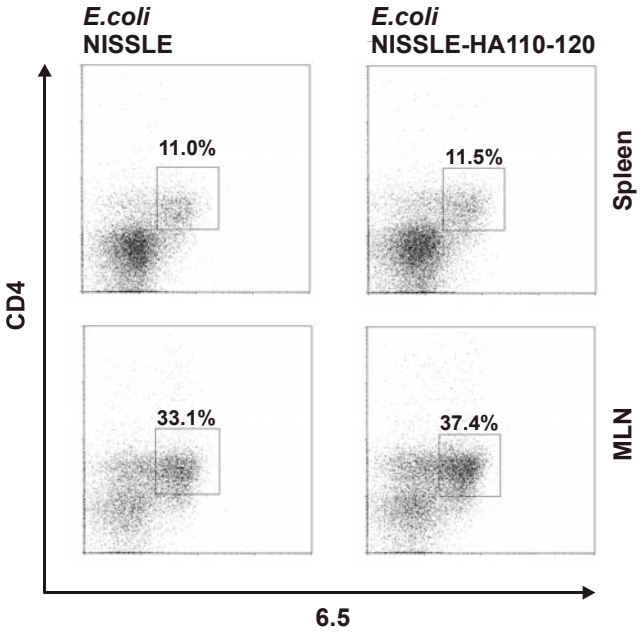


Figure 8: Adoptive transfer of HA-specific $CD4^+$ T cells into bacterial colonized RAG-$1^{-/-}$ mice does not lead to antigen specific clonal expansion of transgenic T cells. RAG-$1^{-/-}$ mice were injected i.p. with 1 x 10^7 6.5^+CD4^+ transgenic T cells and recipients were inoculated with *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After three weeks flow cytometric analysis was performed on spleen and MLN to determine to percentage of 6.5^+CD4^+ transgenic T cells.

3.6 Treatment of RAG1$^{-/-}$ mice reconstituted with HA-specific CD4^{+} T cells with DSS and bacterial colonization did not induce an antigen specific immune response

A variety of mechanisms contribute to the ability of the gut to either react or remain tolerant to antigen present in the intestinal lumen. The epithelial cells form a barrier against exposure to mucosal microflora and other mucosal antigens and thus play a key role in the regulation of mucosal immune responses. Crucial for an efficient barrier function are specialized adaptations of the intestine, including tight junctions between epithelial cells, secretion of mucus, defensins and immunoglobulin (Ig) A. Intestinal epithelial cells can control the uptake, transmission and presentation of antigens through a brought set of pathways. Uptake of noninvasive bacteria across the healthy epithelium can occur only by active vesicular transport across the epithelial cells or by dendritic cells (DC), which actively open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample bacteria (Rescigno et al., 2001). To exclude that the intact epithelial barrier prevents efficient uptake of the *E.coli* NISSLE-HA110-120, experiments were repeated under conditions leading to the disruption of the epithelium. Dextran sulfate sodium (DSS) is a chemical agent which is known to induce colitis in rodents when administrated with the drinking water. DSS-colitis is accompanied with activation of non-lymphoid cells such as macrophages and the release of pro-inflammatory cytokines. The induction of colitis by DSS has been shown to be a T cell independent model for IBD but it is not yet clear if the outcome of colitis influences T cell behavior. To be sure that in this system only HA-specific CD4^{+} T cells can act as effector cells in DSS colitis, adoptive transfers of transgenic T cells into T cell deficient RAG1$^{-/-}$ mice were done (Fig. 9).

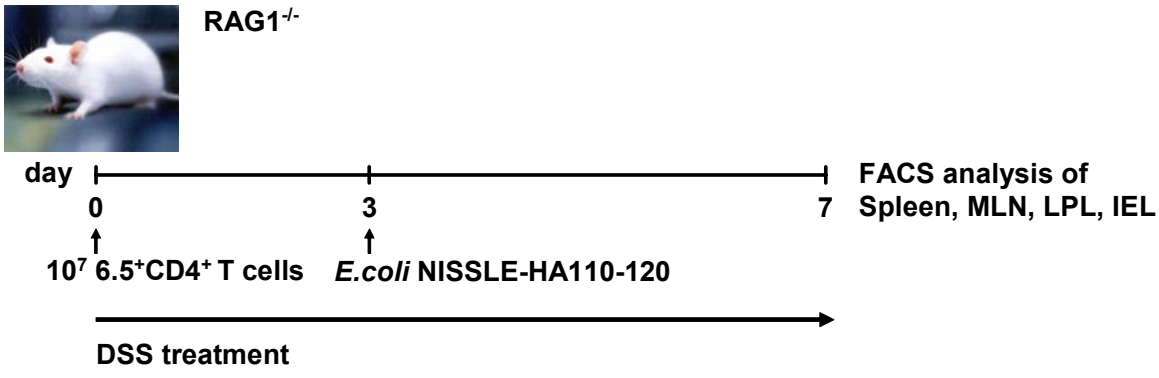


Figure 9: Schematic representation of the experimental course.

$6.5^{+}CD4^{+}$ transgenic T cells enriched from spleen and MLN of TCR-HA mice were transferred i.p. into $RAG1^{-/-}$ mice and recipients were treated with 6% DSS in the drinking water for at least 7 days. At day 3 of DSS treatment the $6.5^{+}CD4^{+}$ recipient mice were fed with *E.coli* NISSLE-HA110-120, control bacteria or PBS. Loss of body weight was measured during the DSS treatment to follow up colitis induction. In contrast to untreated control mice, all DSS treated mice lost 25% of their weight within 7 days of DSS treatment independent of the bacterial strain used for colonization. (Fig. 10).

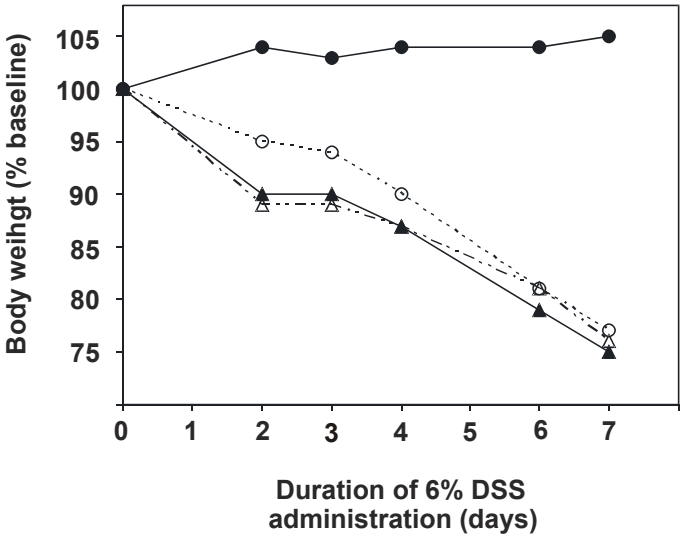


Figure 10: DSS induced colitis in $RAG1^{-/-}$ mice. RAG-$1^{-/-}$ mice were injected i.p. with 10^7 $6.5^{+}CD4^{+}$ transgenic T cells and treated with 6% DSS in the drinking water for 7 days. At day 3 recipients were orally inoculated with PBS (open circles), *E.coli* NISSLE (closed triangles) or *E.coli* NISSLE-HA110-120 (open triangles). Closed circles describe mice that did not receive DSS. Disease severity was measured daily and is expressed in terms of body weight loss.

After one week of DSS treatment spleen, MLN, LPL and IEL were analyzed by flow cytometry for the distribution of $6.5^{+}CD4^{+}$ T cells in the different organs (Fig. 11). The lowest amount of transgenic T cells was found in $RAG1^{-/-}$ mice which were not treated with DSS. In the spleen and MLN of DSS treated mice nearly no differences in the percentage of transgenic T cells were found when comparing mice colonized with *E.coli* NISSLE-HA110-120 and control bacteria. Surprisingly, the number of transgenic LPL was decreased in mice which were colonized with *E.coli* NISSLE-HA110-120 (0.8 %) or *E.coli* NISSLE (1.7 %) in comparison to the control mice (2.5 % and 2.4 %). In the case of IEL there was also a decrease of transgenic cell numbers when inoculating the mice with *E.coli* NISSLE (0.7 %) or *E.coli* NISSLE-HA110-120 (0.7 %) in contrast to

mice receiving DSS (1.6 %) or untreated mice (2.3 %). However, as reduction in cell number was seen with both, *E.coli* NISSLE and *E.coli* NISSLE-HA110-120, it cannot be considered as antigen-specific effect.

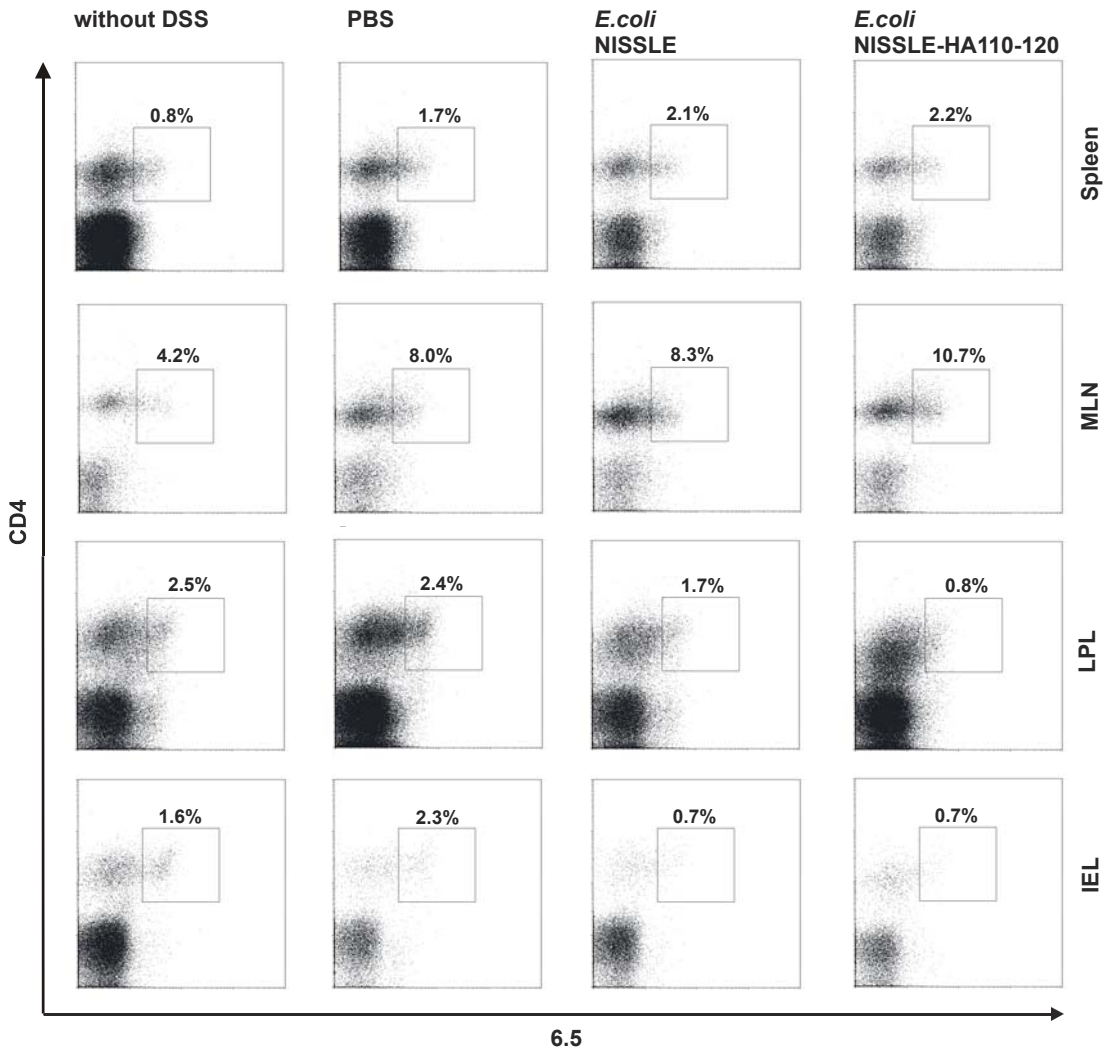


Figure 11: Distribution of HA-specific CD4$^+$ T cells in RAG1$^{-/-}$ mice after adoptive transfer of 6.5$^+$CD4$^+$ T cells, DSS treatment and bacterial colonization. RAG1$^{-/-}$ mice were reconstituted with 6.5$^+$CD4$^+$ T cells, treated with 6 % DSS in the drinking water for a period of 7 days and inoculated at day 3 of DSS treatment with PBS, *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After 7 days the mice were sacrificed and the percentage of transgenic T cells was measured by flow cytometry in spleen, MLN, lamina propria and intestinal epithelium.

For further characterization, the activation status of the recovered transgenic T cells was investigated by CD69 and CD25 measurement in the spleen (Fig. 12A), MLN (Fig. 12B) and LPL (Fig. 12C).

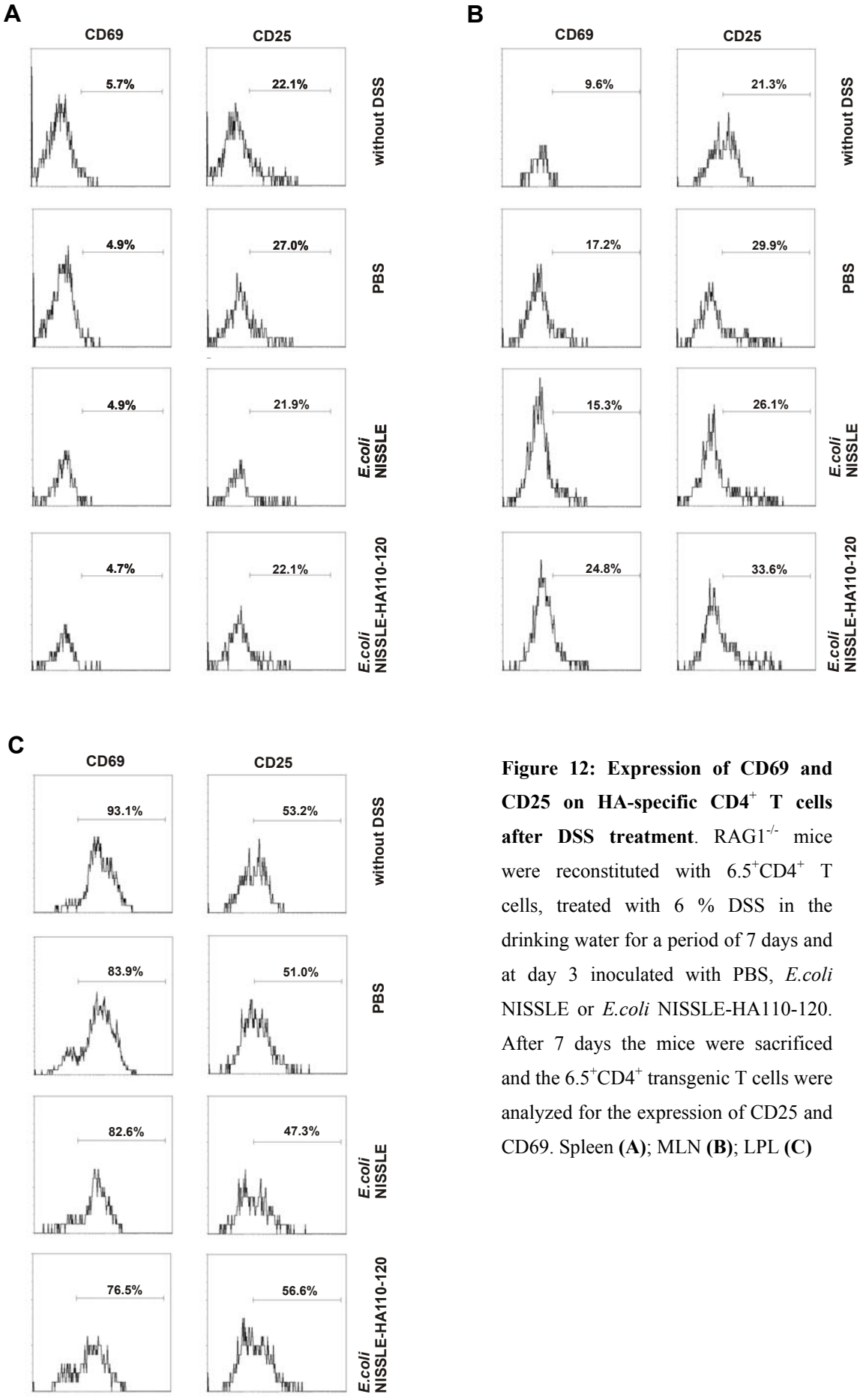


Figure 12: Expression of CD69 and CD25 on HA-specific $CD4^+$ T cells after DSS treatment. $RAG1^{-/-}$ mice were reconstituted with 6.5^+CD4^+ T cells, treated with 6 % DSS in the drinking water for a period of 7 days and at day 3 inoculated with PBS, *E.coli* NISSLE or *E.coli* NISSLE-HA110-120. After 7 days the mice were sacrificed and the 6.5^+CD4^+ transgenic T cells were analyzed for the expression of CD25 and CD69. Spleen **(A)**; MLN **(B)**; LPL **(C)**

Analyzing the transgenic T cells in the spleen, no differences in the activation pattern could be observed. Comparing the expression patterns of MLN between all DSS treated mice and DSS untreated mice revealed that in DSS treated mice there is an increase in the amount of CD69-positive $6.5^{+}CD4^{+}$ T cells (17.2 %, 15.3 %, 24.8 %) compared to untreated mice (9.6 %). This effect was similar for the expression of CD25 on transgenic T cells with 29.9 %, 26.1 % and 33.6 % in mice that received DSS in contrast to 21.3 % in control mice. Nearly no differences in surface marker expression could be found for $6.5^{+}CD4^{+}$ transgenic T cells in the lamina propria.

These results suggested that the differences in the percentage of $6.5^{+}CD4^{+}$transgenic T cells is a consequence of DSS treatment and is not influenced by the bacterial expression of the specific T cell antigen in the gut lumen. Changes in the expression level of the activation markers on transgenic T cells might be driven by the inflammatory environment in the gut due to DSS treatment and seems to be antigen unspecific. Thus, even under disease conditions where the barrier function of the epithelial layer is destroyed, no effect of bacterial antigen expression in the gut on the specific activation of mucosal T cells was observed.

4 Discussion

In nearly all rodent models of colitis studied to date, disease development has been associated with the presence of enteric flora. However, in non of these models the relevant bacterial antigen has been identified, and it has been difficult to define the direct involvement of distinct bacterial species or specific antigens to the onset of disease. The lack of identification of either the antigenic targets or T cell specificities responsible for disease has compromised efforts to understand the pathogenic contribution of the enteric flora. Studies of spontaneous colitis in C3H/He/JBir mice have demonstrated a surprisingly selective immune reactivity to the enteric flora and have suggested that facultative anaerobes such as *E.coli* and Salmonella species were immune stimulatory organisms (Brandwein et al., 1997). Similarly, *Bacteroides spp*. was a common component of bacterial cocktails that induced colitis in germ-free HLA-B27 transgenic rats colonized with defined flora (Rath et al., 1996). *Heliobacter hepaticus* has been proposed as a requisite component of the enteric flora in the CD45RB transfer and IL-$10^{-/-}$ colitis model (Cahill et al., 1997; Kullberg et al., 1998), although this has been discussed controversially (Dielemann et al., 2000). However, the diversity and specificity of the T cell population responsive to these bacteria still needs to be defined in these and other IBD models. The major advantage of the study described here was the use of a model system for characterization of T cell responses to bacterial associated enteric antigens in which the target antigen, the bacterial carrier organism, and the TCR specificity are well defined.

One possible way to achieve effective mucosal immune responses is represented by the secretion or surface presentation of specific antigens by using systems based on those secretory systems which have evolved in pathogens for their own means. A modified variant of the AIDA autotransporter (Benz & Schmidt., 1989; Benz & Schmidt, 1992; Suhr et al., 1996) was used for the efficient surface presentation of the MHC class II HA110-120 epitope by integrating the peptide sequence into an external loop of the core structure of the AIDA translocator. A technical impediment of these studies was to find a bacterial carrier strain that would stably express the HA110-120-AIDA fusion protein *in vivo* and would stably colonize the intestine of the murine host. Earlier studies have shown that it is not easy to obtain long-term colonization with laboratory bacterial strains newly introduced into the normal flora (Fairweather et al., 1990). To this end

E.coli NISSLE 1917 was chosen as carrier organism, as this strain has been shown to be part of the commensal microflora, it is completely apathogenic, it is characterized by its excellent colonization properties in the gut and used as probiotic strain in biological therapy of intestinal disease in human (Blum et al., 1995; Lodinova-Zadnikove & Sonnenborn, 1997). The adoption of AIDA autotransporter extended expression of HA110-120 *in vivo* and a single oral application of transformed *E.coli* NISSLE to mice resulted in a colonization of the mouse gut up to six month. The bacterial surface expression of the HA110-120 epitope was demonstrated by immunolabeling experiments on bacteria isolated from the feces of colonized mice. Additionally, it could be demonstrated that bacteria expressing the HA110-120 epitope could stimulate the proliferation of their cognate T cells *in vitro* as well as *in vivo*. Thus, the level of bacterial HA-110-120 expression was proven to be sufficient to potently induce an immunogenic response of HA-specific $CD4^+$ T cells.

The colonization of TCR-HA mice with *E.coli* NISSLE expressing the HA110-120 epitope or the transfer of naïve 6.5^+CD4^+ into BALB/c and $RAG1^{-/-}$ mice colonized with HA110-120 expressing *E.coli* NISSLE did not result in the induction of a mucosal immune response. In contrast, recent studies implicated, that the reconstitution of SCID and BALB/c mice with naïve $CD4^+$ T cells specific to OVA and colonization of these mice with OVA expressing *E.coli* induced wasting disease (Yoshida et al., 2001 Yoshida et al., 2002). Consistent with the results obtained in the TCR-HA/*E.coli* NISSLE-HA110-120 model, Iqbal et al. (2002) published a colitis model based on the use of OVA-producing *E.coli* and the transfer of T_H1 or T_H2 OVA-specific $CD4^+$ T cells into $RAG2^{-/-}$ mice. By the transfer of naïve OVA-specific $CD4^+$ T cells no colitis was observed. The reason for the lack of immune response in recipients of naïve HA-specific T cells colonized with *E.coli* NISSLE-HA110-120 is not yet clear, as Yoshida et al. (2001) could demonstrate activation of T cells and induction of intestinal inflammation in the OVA-system. Differences in the protocol used could be a reason for the different outcome of the experimental results. Yoshida et al. (2001) used SCID mice instead of $RAG1^{-/-}$ mice as recipients and the number of transferred T cells (10^7) was comparably high. Naïve T cells need a certain antigen threshold for the induction of an immune response against specific antigen. To exclude that the antigenic yield produced by *E.coli* NISSLE-HA110-120 in the gut of recipient mice was too low to activate naïve T cells, *in vitro* activated 6.5^+CD4^+ T cells were adoptively transferred into colonized BALB/c

mice to abate the stimulus threshold. However, also activated transgenic T cells with an abated antigen threshold were not able to respond to the luminal bacterial associated antigen, neither with clonal expansion nor with changes in the activation pattern of the $6.5^{+}CD4^{+}$ T cells (Fig. 7).

Several mechanisms contribute to the ability of the gut to either react or remain tolerant to antigen present in the intestinal lumen. The epithelial cells form a barrier against exposure to mucosal microflora and other mucosal antigens. The internalization of dietary antigens appears to be widespread throughout the intestinal epithelium, and is carried out by epithelial cells (Mowat & Viney, 1997), whereas the uptake of bacteria mainly occurs in the Peyer's patches, via M cells (Neutra et al.,1996). Although only invasive bacteria can efficiently induce their own phagocytosis through M cells, noninvasive bacteria have been shown to enter the epithelium by active vesicular transport across the epithelial cells or by denditric cells which open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample bacteria from the gut lumen (Rescigno et al., 2001). However, these mechanisms of bacterial uptake are rather inefficient. Inefficient transepithelial transport of luminal bacteria could be the reason for unresponsiveness against bacterial derived HA110-120 peptide in this described model. Therefore, the epithelium of $RAG1^{-/-}$ mice was disrupted by DSS treatment (Okayasi et al., 1990) before oral application of *E.coli* NISSLE-HA110-120. DSS induced tissue disintegration resultes in increased luminal bacterial translocation and thereby exposure of the mucosal immune system to bacterial associated antigens. All DSS treated $RAG1^{-/-}$ recipients lost about ~ 15 to 25 % of body weight within 7 days, indicating an active colitis (Fig. 10). Also changes in the percentage and the activation status of transgenic T cells was observed. However, these effects were not antigen specific, the differences between the experimental groups seemed to be a consequence of DSS treatment and were not influenced by the bacterial antigen expression. Furthermore, the application of *E.coli* NISSLE 1917 did not ameliorate the severity of intestinal inflammation, suggesting that this probiotic bacterial do not have a positive effect in DSS induced colitis.

One possible reason for the unresponsiveness of T cells against luminal *E.coli* NISSLE-HA110-120 could be an organ specific failure to produce the correct T cell epitope HA110-120, thereby preventing efficient MHC class II presentation of the antigenic

determinant at the surface of antigen presenting cells in the intestine. The uptake of the transformed bacteria normally occurs by an active transport into the epithelial cells or by dendritic cell, which send dendrites outside the epithelium to sample the bacteria. Within the APC bacterial products are degraded in acidic compartments resulting in the generation of antigenic peptides. For MHC class I peptides, Kuckelkorn et al. (2002) could demonstrate that proteasomes in the small intestine generate a specific set of MHC class I restricted epitopes and that proteasomes derived from other organs produce a distinct peptide pattern due to their organ-typic subunit composition. They figured out, that proteasomal antigen processing could be a further step in the control of organ specific immune response. As the processing of *E.coli* NISSLE-HA110-120 by peritoneal macrophages resulted in specific T cell stimulation *in vitro* and *in vivo*, it might be possible that the kind of MHC class II epitopes generated in the lysomal compartments of APC from the peritoneum differ from those in the small intestine.

As different experimental approaches used in this thesis and they all did not result in a measurable immune response of HA-specific $CD4^+$ T cells against the bacterially expressed specific HA110-120 peptide in the mouse intestine, *E.coli* NISSLE 1917 seems to be a useful carrier strain for localized delivery of specific molecules in the intestine in cases, where an immune response against the carrier strain and its products is undesired. As demonstrated in VILLIN-HA x TCR-HA transgenic mice mucosal lymphocytes are able to intervene in an inflammatory process by the production of surface molecules or the secretion of cytokines with regulatory properties. To use this knowledge of intestinal regulatory mechanism, the scope of *E.coli* NISSLE 1917 probiotic action could be extended by genetic modification of these bacteria to deliver anti-inflammatory or other biological important molecules to the inflamed mucosa. Food-grade *Lactocossus lactis* has been engineered to secrete IL-10 and was therapeutically effective when given intragastrically to mice suffering from IBD (Steidler et al., 2000). They had to inoculate the mice daily with a high dose of transformed bacteria to ensure intestinal colonization. *E.coli* NISSLE 1917 colonization is efficient to such a degree that one oral application resulted in intestinal colonization and stable antigen expression for up to two weeks without further addition of antibiotics. Combining the excellent colonization properties and a non immunogenic character, this strain is predestinated to be used as a carrier organism for gut focused drug specific therapy of IBD. Cost effective localized delivery of a therapeutic agent that is actively

synthesized in situ by food-grade bacteria may have potential clinical applications for treatment of intestinal inflammation, particularly as an alternative to systemic treatment. In principle, this method may also be useful for intestinal delivery of other protein therapeutics that are unstable *in vivo* or difficult/expensive to produce in large quantities.

5 Summary

In this study the influence of luminal bacterial antigen on the development of a mucosal T cell response in the mouse intestine was investigated. Bacterial autotransporter proteins represent an extremely useful system for the efficient surface presentation or secretion of heterologous antigens by Gram-negative bacteria in the intestine. A modified variant of the AIDA autotransporter was used for the efficient surface presentation of influenza virus A/PR/8/34 hemagglutinin (HA) MHC class II epitope HA110-120. The antigenic determinant HA110-120 was detectable at the surface of the carrier strain *E.coli* NISSLE 1917. This apathogenic strain is part of the commensal microflora of the gut and is characterized by its excellent colonization properties. It could be shown that bacterial autodisplay of the antigenic HA110-120 peptide by *E.coli* NISSLE 1917 led to the efficient stimulation of HA110-120 specific T cell proliferation *in vitro* and *in vivo*. However, following oral application of the NISSLE-HA110-120 to TCR-HA mice, in any case, no significant effect of bacterial antigen expression in the gut on migration, clonal expansion and the activation status of HA-specific $CD4^+$ T cells could be observed. Moreover, the adoptive transfer of naïve and *in vitro* activated HA-specific $CD4^+$ T cells into BALB/c and $RAG1^{-/-}$ mice colonized with HA-expressing *E.coli* NISSLE did not induce clonal expansion and had no effect on the activation status of HA-specific T cells. Furthermore, DSS treatment of $RAG1^{-/-}$ mice to disturb the epithelial barrier before reconstitution with HA-specific $CD4^+$ T cells and colonization with *E.coli* NISSLE-HA110-120, did not result in an effect of bacterial antigen expression in the gut on the specific activation of mucosal T cells. Under these experimental conditions the mucosal immune system did not respond to the specific bacterial associated antigen independent of a healthy or disrupted epithelial barrier. Due to the excellent colonization properties of *E.coli* NISSLE 1917, the complete unresponsiveness of T cells against these bacteria, this strain is predestined to be used as a carrier organism for gut focused drug specific therapy of IBD. This system will be an extremely useful tool for the localized delivery of anti-inflammatory molecules or other biological important molecules to the inflamed mucosa.

CHAPTER IV

Materials and Methods

Materials and Methods

1 Mice

BALB/c mice were obtained from Harlan (Borchen, Germany) and RAG-1$^{-/-}$ mice from Jackson (USA). TCR-HA transgenic mice expressing a TCR αβ specific for the peptide 110-120 from influenza HA presented by I-E ^{d} have been described previously (Kirberg et al., 1994). VILLIN-HA transgenic animals were generated using a construct containing the villin promoter to direct expression of the influenza virus A/PR8/34 hemagglutinin to epithelial cells along the entire crypt-villus axis (Pinto et al., 1999), a 9 kB regulatory domain (construct kindly provided by Sylvie Robine, Institut Curie, Paris, France) and the complete HA-sequence. Transgene expression was analyzed by PCR screening on genomic tail DNA. PCR was performed using a villin specific 5' primer (5'-CCT TAA GCC GGC TGT GAT AG-3') and a HA specific 3' primer (5'-TTA CTA TTA GAC GGG TGA TGA TGA ATA-3'). RAG1$^{-/-}$, TCR-HA and VILLIN-HA mice were bred in the animal facility at the German Research Centre for Biotechnology. Mice aged 12 to 16 weeks were used for experiments which were all performed according to National and Institutional Guidelines. Extensive microbial and serological studies were performed to exclude the presence of pathogenic bacteria, viruses, fungi and parasites which could potentially cause mucosal inflammation in these mice. No pathogens could be detected in all clinical samples studied.

2 Preparation of lymphocyte populations

Spleens were rinsed with erythrocyte lysis buffer (Qiagen, Hilden, Germany). Mesenteric lymphnodes (MLN) were disaggregated by passing through a 100 µm mesh. Cells were washed with FACS buffer (PBS, 2% FCS, 2mM EDTA) and collected by centrifugation. LPL were isolated from the small intestine as described (Guy-Grand et al., 1978). Briefly, after flushing the gut with PBS, the Peyer's patches were removed. The gut was opened longitudinally and cut into small pieces. Mucus and epithelial layer were removed by stirring at 37 °C, first, two times for 10 min in 60 ml of PBS containing 3 mM EDTA, then twice for 15 min in 30 ml of Ca-free RPMI containing

1% FCS, 1 mM EGTA and 1.5 mM $MgCl_2$. Gut pieces were collected, vortexed for 20 sec before finely mincing the tissue. Finally LPL were released by enzymatic digestion of the gut at 37°C for 90 min in 30 ml RPMI containing 20 % FCS and 100 U/ml collagenase. To improve tissue disintegration the suspension was dissociated by multiple aspirations through a syringe after 45 min and the end of the incubation. The cell suspension was passed through a 100 µm mesh and centrifuged for 10 min at 1200 rpm. LPLs were collected by density centriguation using a ficoll gradient.

IEL from the small intestine were isolated as described previously (Guy-Grand et al. 1978). In brief, Peyer's patches were removed and, after flushing with PBS, the gut was opened longitudinally. The mucosa was scraped off with a scalpel and then dissociated by stirring in 50 ml RPMI containing 10 % FCS and dithioerythritol (1 mM) for 15 min at 37°C. Cells were collected by centrifugation and the pellet was vortexed for 3 min in HANKS medium containing 10% FCS. 2 x 40 ml were rapidly passed through a glass wool column (1.5 g packed in a 20 ml syringe; Fisher Scientific), previously equilibrated with HANKS/5 mM Hepes. The eluate was collected, centrifuged and cells were resuspended in FACS buffer.

3 Antibodies and flow cytometry

The monoclonal antibody 6.5 (α-TCR-HA) was purified from hybridoma supernatant and was used in fluorescein isothiocyanate (FITC)-labeled or biotinylated form. Monoclonal antibodies α-CD4 (GK1.5 and L3T4), α-CD25 (PC61), α -CD45RB (16A), α-CD62L (MEL-14), and α-CD69 (H1.2F3) were used as biotin, FITC or phycoerythrin (PE) conjugates. PE-streptavidin- or APC-(Allophycocyanin)-streptavidin-conjugates were used as secondary reagents (BD Bioscience, San Jose, CA). Two- and three color flow cytometry was performed on a FACSCalibur (BD Bioscience). Data were analyzed with CellQuestPro software (BD Biosciences). For gene expression profiling 6.5^+CD4^+ T cells were sorted with the MoFlow cells sorter (Cytomation, Fort Collins, CO).

4 Carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling of lymphocytes

Cell suspension were prepared as described above. Cells were washed in RPMI without FCS, resuspended at a concentration of 10^7 lymphocytes / ml and incubated with 2.5 µM CFSE (Molecular Probes, Göttingen, Germany) for 8 min at 37 °C. Two volumes of FCS were added and cells were incubated for additional 5 min at 37 °C. After CFSE labeling the cells were washed twice with PBS to remove excess of CFSE and FCS.

5 Adoptive transfer

For adoptive transfer experiments in BALB/c, $RAG1^{-/-}$ or VILLIN-HA mice, red blood cell-depleted splenocytes from TCR-HA mice were enriched by AutoMACS using the $CD4^+$ T cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturers instructions. In case $CD25^+$ cells should be depleted from the $CD4^+$ T cell population, biotinylated α-CD25 antibody was added to the biotin antibody cocktail of the $CD4^+$ T cell Isolation Kit. The percentage of 6.5^+CD4^+T cells was determined by flow cytometry analysis as described above. Enriched transgenic cells, either unlabeled or CFSE labeled, were injected (i.p.) into age and sex matched BALB/c, $RAG1^{-/-}$ or VILLIN-HA mice.

For transfer of activated transgenic T cells splenocytes isolated from TCR-HA mice were stimulated *in vitro* with 10 µg/ml HA110-120 peptide and cultured for 4 days. Dead cells were removed by ficoll gradient and viable cells were cultured for an additional day. Flow cytometric analysis were performed to determine the percentage of 6.5^+CD4^+ lymphocytes and the activation status of the HA specific $CD4^+$ T cells was characterized by staining with α-CD25 and α-CD69.

6 Histology

Mice were sacrificed and the gut was immersion fixed in buffered formalin, embedded in paraffin, sectioned at 4 µm thickness and stained with hematoxylin and eosin (H&E). Immunohistochemistry for T lymphocytes was performed using the rat-anti-human-CD3

antibody clone CD3-12 (Serotec Ltd., Kidlington, UK) at 1:1.600 dilution and the avidin-biotin-complex (ABC) method with diaminobenzidin as chromogen. Immunohistochemistry sections were counterstained with hematoxylin.

7 Proliferation assay

For antigenic stimulation of $6.5^{+}CD4^{+}$ T cells from spleen and MLN 5 x 10^5 cells were plated in 96-well flat-bottom plates in a final volume of 200 µl IMDM-medium containing 10 % FCS. Flow cytometry analysis was performed to normalize the number of specific $6.5^{+}CD4^{+}$ lymphocytes in bulk-cultures. Cell suspensions were incubated in the presence or absence of 10µg/ml HA peptide (SSFERFEIFPK) (Hackett et al., 1983) at 37°C. ^{3}[H]-thymidine incorporation over the last 15 h of a 48 h culture was measured by scintillation counting. In case intestinal lymphocytes were used as responders, 10^5 IEL or LPL were cultured with different amounts of the HA peptide and 5 x 10^5 irradiated BALB/c splenocytes as feeder cells. Culture supernatants were collected for CBA measurement after 48 h and proliferation of the cells was estimated by culturing the cells in the presence of 1 µCi ^{3}[H] thymidine per well for additional 16 h.

8 Cytokine bead array (CBA)

Quantification of cytokines in culture supernatants of stimulated *versus* non stimulated lymphocytes (see proliferation assay) was performed using the CBA kit (Becton Dickinson Heidelberg, Germany) following the manufacturers recommendations. Briefly, polystyrene beads (7.5 µm diameter) stained to 6 different fluorescence intensities, which have an emission wavelength of ~650 nm (FL 3), were coupled with antibodies to different cytokines contained in the kit. The captured cytokines are then detected using six specific antibodies coupled to phycoerythrin (PE), which emits its flourescence at ~585 nm (FL 2). Standardized mixtures of all cytokines served as internal controls. 50 µl of culture supernatant or cytokine standard were added to a mixture of 50 µl each capture antibody-bead reagent and detector antibody-PE reagent, respectively. The mixture (150 µl in total) was incubated for 2 hours at room temperature. Unbound detector antibody-PE reagent was removed by a single washing

step before data acquisition was performed by flow cytometry using a FACSCalibur. Acquired data were analyzed using the Becton Dickinson Cytometric Bead Array software.

9 DNA microarray hybridization and analysis

Total RNA from sorted 6.5^+ $CD4^+$ T cells was isolated using the RNAeasy kit (Qiagen, Hilden, Germany). Quality and integrity of total RNA isolated from 10^5 sorted T cells was assessed by running all samples on an Agilent Technologies 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). For RNA amplification the first round was done according to Affymetrix without biotinylated nucleotides using the Promega P1300 RiboMax Kit (Promega, Mannheim, Germany) for T7 amplification. For the second round of amplification the precipitated and purified RNA was converted to cDNA primed with random hexamers (Pharmacia, Freiburg, Germany). Second strand synthesis and probe amplification were done as in the first round with two exceptions: an incubation with RNAse H preceeded the first strand synthesis to digest the aRNA, and the T7T23V oligo for initiation of the second strand synthesis was used. 12.5 µg biotinylated cRNA preparation was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MG-U74Av2 chips for 16 hours. After hybridization, GeneChips were washed, stained with streptavidin-PE and read using an Affymetrix GeneChip fluidic station scanner. Analysis was done with gene expression software (GeneChip, MicroDB, and Data Mining Tool, all Affymetrix). The entire data set of this micro-array experiment is in MIAME-format and accessible online under www.gbf.de/array/download.

10 Generation of the HA110-120 expression plasmid and the bacterial strain *E.coli* NISSLE-HA110-120

As carrier protein for the MHC class II HA110-120 peptide the AIDA autotransporter vector which was kindly provided by M. Alexander Schmidt (University Münster, Germany) was used. The plasmid harboring the AIDA autotransporter is an ampicillin resistant pBR322 derivate that expresses a recombinant AIDA protein under the control of its natural promoter (Benz & Schmidt, 1989). The AIDA encoding sequence was modified to remove the native passenger; it consists of signal peptide, a linker region incorporating a multiple cloning site, and the entire β-barrel core AIDAc. To internally integrate the MHC II 110-120 peptide from influenza HA into the n-terminal AIDAc the vector was digested enzymatically with XbaI, dephosphorylated and ligated to the double-stranded oligonucleotide sequence of HA110-120 VSSFERFEIFPKESS (5'-CTA GCC GTG TCA TCA TTC GAA AGA TTC GAA ATA TTT CCC AAA GAA AGC TCA-3'). The insertion was confirmed by DNA sequencing. As bacterial antigen carrier the *E.coli* NISSLE 1917 strain was used. Bacteria were kindly provided by Florian Gunzer (Hanover Medical School, Hanover, Germany). Bacteria were routinely stored with 40% glycerol in Luria-Bertani (LB)-medium at -70°C and grown in LB liquid culture or on LB agar plates containing 100µg/ml ampicillin.

11 Immunofluorescence

For immunofluorescence staining, bacteria expressing the HA110-120/AIDA autotransporter fusion protein and *E.coli* NISSLE and control bacteria were cultured at 37 °C to an OD_{600} of 0.4. 1 ml of the suspension was centrifuged and the bacterial pellet was incubated in 1 % BSA/PBS for blocking unspecific antibody binding. Bacteria were washed with PBS, incubated for 1 h at RT with the mouse α-HA110-120 antibody CMI1.2. followed by washing twice with PBS. Bacteria were resuspended in 1 ml PBS and 25 µl of the suspension were spread and air dried on circular coverslips (15 mm diameter). Centrifugation was carried out at 1500 x g for 5 min at 4 °C. Labeled bacteria were fixed in 3.7 % paraformaldehyd in PBS for 20 min and afterwards washed two times with PBS. The IgG CyTM3 conjugated goat anti mouse secondary antibody (Dianovo, Hamburg, Germany) was added and after 1 h incubation at 37°C in a

humidified chamber the coverslips were washed with PBS three times. The cover slips were subsequently mounted in Fluoprep (bioMérieux, Marcy l'Etoile, France) and analyzed using a Zeiss Axiophot microscope.

12 Immunogenicity of *E.coli* NISSLE-HA-110-120

For *in vitro* studies peritoneal macrophages from BALB/c were isolated and 2 x 10^4 cells/well were cultured over night in antibiotic free medium in 96-well flat-bottom plates. Non adherent cells were removed and indicated numbers of viable *E.coli* NISSLE and *E.coli* NISSLE-HA110-120 were added to the macrophages. After 5 h 4 x 10^5 CD19 depleted splenic cells from TCR-HA mice were added in medium containing 200 µg/ml streptamycin, 200 U/ml penicillin and 100 µg/ml gentamycin to kill to viable bacteria. 24 h later proliferative response of HA specific T cells was determined by adding 1 µCi of ^{3}[H]-thymidine per well for the final 18 h of the experiment. Thymidine incorporation was measured by scintillation counting.

To measure the capacity of recombinant *E.coli* expressing HA110-120 peptide to stimulate their corresponding HA-specific $CD4^+$ T cells *in vivo,* the adoptive transfer system was used. Cells were prepared from the spleen of TCR-HA mice and HA-specific T cells were enriched by $CD4^+$ T cell Isolation Kit (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) as described above. The percentage of transgenic T cells was measured by Flow cytometry analysis and enriched $CD4^+$ T cells were labeled with CFSE before adoptive transfer. BALB/c mice were injected i.p. with 2.5 x 10^6 CFSE labeled transgenic T cells and 24 h later the recipient mice were injected i.p. with either PBS, 10^8 *E. coli* NISSLE as negative control or 10^8 *E. coli* NISSLE-HA110-120. Two days later splenocytes and MLN cells were isolated and the proliferation of transgenic T cells was measured by loss of CFSE labeling.

13 Colonization of mice with the *E.coli* NISSLE-HA110-120

Bacterial colonization of the gut was performed by oral application of *E.coli* NISSLE and *E.coli* NISSLE-HA110-120. To ensure stable colonization for more than one week 0.3 mg/ml ampicillin were added to the drinking water. Successful colonization was checked by plating mouse feces on plates containing 100 µg/ml ampicillin during the time of the experiment.

14 Induction of colitis

$6.5^{+}CD4^{+}$ transgenic T cells enriched from spleen and MLN of TCR-HA mice were transferred i.p. into $RAG1^{-/-}$ mice. Recipient mice were fed 6 % (wt/vol) Dextran sodium sulfate (DSS) (molecular weight, 40 kDa; ICN Biomedicals Inc., Aurora, Ohio, USA) dissolved in water for 7 days to induce acute colitis. At day 3 of DSS treatment the $6.5^{+}CD4^{+}$ recipient mice were fed with *E.coli* NISSLE-HA110-120, control bacteria or PBS. Loss of body weight was measured during the DSS treatment to follow up colitis induction. No mortality was observed during the 7 days of DSS administration.

CHAPTER V

Abbreviations

References

Abbreviations

AICD	Activation induced cell death
AIDA	Adhesin involved in diffuse adherence
APC	Antigen presenting cell
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
CD	Crohn's disease
CFSE	5'-Carboxyfluorescein diacetat succinimidylester
CFU	Colony forming units
cpm	Counts per minute
CXC	Chemokine ligand
DC	Dendritic cell
DSS	Dextran sodium sulfate
FACS	Fluorescence activated cell sorter
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissue
HA	Hemagglutinin
HEV	High endothelial venules
i.p.	Intraperitonal
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LP	Lamina propria
LPL	Lamina propria lymphocyte
LPS	Lipopolysaccharid
LT	Lymphotoxin
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex

MLN	Mesenteric lymph node
OVA	Ovalbumin
PCR	Polymerase chain reaction
PTG	Prostaglandin
RAG	Recombinase activation gene
SCID	Severe combined immunodeficiency
SED	Subepithelial dome
SP	Signal peptide
TBNS	Trinitrobenzene sulfonic acid
TCR	T cell receptor
TGF	Tumor growth factor
T_H cell	T helper cell
TNF	Tumor necrosis factor
Tnfrs	Tumor necrosis factor receptor family
T_{reg}	Regulatory T cell
UC	Ulcerative colitis

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